**T.C.**

**CANAKKALE ONSEKIZ MART UNIVERSITY**

**SCHOOL OF GRADUATE STUDIES**

**DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

# CHARACTERIZATION OF PITAYA *N*-LINKED GLYCANS AND *IN-VITRO* IDENTIFICATION OF POTENTIAL PREBIOTIC EFFECT

**MASTER OF SCIENCE THESIS**

**MELDA KARYELİOĞLU**

**Thesis Supervisor**

**ASSOC. PROF. SERCAN KARAV**

**ÇANAKKALE – 2023**

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The study titled "Characterization of Pitaya *N*-Linked Glycans and *In-vitro* Identification of Potential Prebiotic Effect", prepared by Melda KARYELİOĞLU under the direction of Assoc. Prof. Sercan KARAV and presented to the following jury members on 15/06/2023 was unanimously accepted **as a MASTER THESIS** at Canakkale Onsekiz Mart University, School of Graduate Studies, **Department of Molecular Biology and Genetics.**

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20/06/2023

## ETİK BEYAN / ETHICAL STATEMENT

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Melda KARYELİOĞLU
15/06/2023

**ACKNOWLEDGEMENT**

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Melda Karyelioğlu

Çanakkale, June 2023

**PİTAYA *N*- BAĞLI GLİKANLARININ KARAKTERİZASYONU VE POTANSİYEL PREBİYOTİK ETKİSİNİN *IN-VITRO* KOŞULLARDA BELİRLENMESİ**

# ÖZET


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Çanakkale Onsekiz Mart Üniversitesi

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15/06/2023, 52



Hylocereus cinsi bir kaktüs meyvesi olan Pitaya, zengin içeriğe ve fonksiyonel bileşiklere sahiptir. Pitaya, son zamanlarda dünyada oldukça popüler bir meyve haline gelmiştir. İnsan sağlığı üzerinde kanser kemoprevansiyonu, anti-inflamatuar özellik, antidiyabetik etki gibi olumlu etkileri olan pitaya, içerdiği oligosakkarit (glikanlar) yapıları de insan bağırsak sağlığı için önemli bir meyve niteliğindedir. Doğada serbest veya bağlı formda bulunabilen glikanların insan sağlığı üzerinde önemli etkileri bulunmaktadır. İnsanlarda bu glikanları parçalayabilecek özgüllükte glikozidaz enzimi bulunmadığından glikanlar bozulmaya uğramadan, kolona kadar ulaşabilirler. Bu glikanlar mikrobiyotadaki bazı simbiyotikler tarafından tek karbon kaynağı olarak kullanılabilmekte olup, seçici gelişime olanak sağlamaktadırlar. Özellikle insan mikrobiyotasında bulunan Bifidobacterium infantis bu glikanları tüketilebilecek özelliklere sahiptir. Salgıladığı Endo-*β-N*-asetilglukozaminidaz (EndoBI-1) enzimi ile *N*-bağlı glikanları serbestleştirerek karbon kaynağı olarak kullanabilir. Pitayada bulunan serbest glikanlar, yüksek prebiyotik etkiye sahipken, bağlı glikanların biyolojik özellikleri ile alakalı çalışmalar sınırlıdır.

Bu amaçla; pitaya örneklerinin öncelikle protein içeriği araştırılmıştır. Pitaya örneklerinden izole edilen protein miktarı, florometrik yöntem kullanılarak kantifikasyonu sağlanarak elde edilen proteinler görüntülenmiştir. Sonrasında *N*-glikanlar özgün EndoBI-1 ve PNGaz-F enzimleri ile kesilip saflaştırılmıştır. Elde edilen glikanlar, detaylı bir şekilde

MALDI-TOF-MS analizi ile karakterize edilmiştir. Saflaştırılan glikanların prebiyotik etkisinin incelemek amacıyla *in-vitro* sindirim modeli kullanılarak sindirilebilirliği belirlenmiş ve seçilen probiyotik bakteriler tarafından kullanımı değerlendirilmiştir.

Bu çalışmanın gerçekleştirilmesiyle üretimi ve kullanımı hızla gelişen pitaya meyvesi için *N*-glikan profili ve prebiyotik potansiyeli aydınlatılmıştır. Böylece pek çok fonksiyona sahip olduğu bilinen glikanların karakterizasyonu yapılarak, literatüre katkı sağlanmıştır. Çalışma sonucunda ortaya çıkan serbestleşmiş pitaya N-glikanlarının prebiyotik özelliğinin gösterilmesi ile yeni AR-GE çalışmalarının önü açılmış olacaktır.

**Anahtar Kelimeler:** Pitaya, *N*-Glikan, *In-vitro* sindirim, Prebiyotik

**CHARACTERIZATION OF PITAYA *N*-LINKED GLYCANS AND *IN-VITRO* IDENTIFICATION OF POTENTIAL PREBIOTIC EFFECT**

**ABSTRACT**

Melda KARYELİOĞLU
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Master of Science Thesis in Molecular Biology and Genetics

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15/06/2023, 52

Pitaya belongs to the Cactaceae family from the genus Hylocereus. Global cultivation and consumption of pitaya have lately increased. Pitaya plays critical roles in human health, including cancer chemoprevention, anti-inflammatory, and anti-diabetic actions. Studies show that dragon fruit is beneficial to human gut health due to its high oligosaccharide content. These glycans can exist in both free or linked forms. When it is consumed by humans, glycans can get into the colon in an undigested form due to the lack of glycosidase enzymes in humans. Here, they can be utilized as a prebiotic carbon supply by microorganisms present in the gastrointestinal tract and stimulate selective growth. Studies show that pitaya oligosaccharides have a probiotic effect on some microorganisms. However, the bound glycan structures of the fruit are not clearly investigated. Due to rare research on conjugated glycans, released *N*-linked glycans from pitaya, possibly have a significant prebiotic influence on the gut.

This study conducted research on the glycoproteins found in pitaya. Pitaya samples from two different locations were tested and visualized for protein content. A unique glycosides enzyme was used to liberate *N*-glycans from the glycoproteins they attached. Characterization of novel *N*-glycan structures was analyzed through MALDI-TOF-MS. The pure glycans' prebiotic effect was evaluated using an in-vitro digesting model.

As a result, we enlighten the information regarding pitaya *N*-glycan composition and prebiotic conduct of bioactive *N*-glycans. In this context, it has been explained how diverse glycoprotein-containing products affect the digestive system and shape the microbiome.

**Keywords:** Pitaya, *N*-Glycan, *In-vitro* Digestion, Prebiotic

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## SYMBOLS and ABBREVIATIONS

| | |
|---|---|
| 2-AA | 2-Aminobenzoic Acid |
| % | Percent |
| ANOVA | Analysis of Variance |
| Asn | Asparagine |
| $dH_2O$ | distilled water |
| et. al | Others |
| EndoBI-1 | Endo-β-*N*-acetylglucosaminidase |
| g | Gram |
| GI | Gastrointestinal |
| h | hour |
| HexNAc | *N*-acetylglucosamine |
| Kg | Kilogram |
| LB | Lysogeny broth |
| M | Molar |
| MALDI-TOF MS | Matrix-Assisted Laser Desorption-Ionisation-Time of Flight Mass Spectrometry |
| mg/mL | Milligram per milliliter |
| min | minute |
| OD | Optical Density |
| pH | Power of Hydrogen |
| PNGase-F | Peptidyl *N*-Glycosidase F |
| RPM | Revolutions per minute |
| s | second |
| SDS-PAGE | Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis |
| Ser | Serine |
| SGF | Simulated Gastric Fluid |
| SIF | Simulated Intestinal Fluid |
| SSF | Simulated Salivary Fluid |
| Thr | Threonine |

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# CHAPTER 1
# INTRODUCTION

## 1.1. Pitaya, Content and Prebiotic Effect

Pitaya or dragon fruit belongs to *Hylocereus* cactus family native to Southern Mexico and Central America (Ortiz-Hernández and Carrillo-Salazar, 2012). Since pitaya has a very interesting color and taste, it has become a popular fruit with increased production in many parts of the world. Today, it is commercially produced in countries such as the Bahamas, Vietnam, Malaysia, Taiwan, Thailand, Indonesia, southern Florida, Israel, Mexico, Japan, and Bermuda (Mercado-Silva, 2018). In Türkiye, pitaya, which has started to be grown in the Mediterranean and Aegean coasts in recent years, is a fairly new fruit for our country.

The most common pitaya species are Hylocereus undatus, which has a pinkish outer shell and a white inner part, and Hylocereus polyrhizus, which has a pinkish outer shell and a red inner part. All pitaya species have black tiny edible seeds resembling kiwi seeds inside. Pitaya has a wide variety of nutritional content and functional compounds (Liaotrakoon, 2013). It is a source of beta carotene, lycopene, vitamin E, fiber, pectin, and polyphenol in terms of content. On the other hand, its rich nutritional content shows antiproliferative effect and a high degree of antioxidant properties against various free radicals (Wichienchot et al., 2010). Dietary fibers, which are abundant in dragon fruit, have prebiotic potential and play a role in regulating metabolic health by promoting the growth of beneficial bacteria in the gut. Dietary fibers are found at an average of 69.3% in a whole pitaya, 56.5% in the peel part of the fruit (Stintzing et al., 2003).

Pitaya also contains minerals such as potassium, phosphorus, sodium, and magnesium in significant amounts. The amount of these minerals in pitaya is higher, especially when compared to other similar fruits mango and pineapple (Stintzing et al., 2003). Vitamins such as vitamin E (33 mg/100 g) and vitamin B3 (0.2-2.8 mg/100 g) are also found in high concentrations in this fruit. In addition, the seeds of pitaya are very rich in essential fatty acids that the body needs and cannot synthesize (Ariffin et al., 2009). The reason why pitaya has drawn a lot of attention in recent years is not only because of its striking appearance, but also because of its high health-supporting properties such as cancer

chemoprevention, and antidiabetic effects, anti-inflammatory properties, and pitaya has also been proven to reduce the risk of cardiovascular disease. According to these studies in the literature, pitaya has the potential to be used as a source of functional components to provide nutrients that can prevent nutrition originated diseases and increase the mental and physical health of consumers (Wichienchot et al., 2010).

Sugars found in pitaya intensely are glucose (Glc) followed by fructose (Liaotrakoon, 2013). These sugars are found in pitaya at a ratio of 2-6 g / 100 g. In saccharide content of fruit, it is known that the free oligosaccharides (~ 90g / kg) in pitaya consist of some fructooligosaccharides (Liaotrakoon, 2013). Free oligosaccharides are structures consisting of 3-10 short-chain monosaccharides. These structures, also known as glycans, are mostly found on the cell surface in eukaryotes and have many functions such as cell growth and development, immune recognition and response, cancer cell metastasis, anticoagulation, intercellular communication, protein folding, molecular mimicry and initiation of microbial pathogenesis (Varki et al., 2017). Fructooligosaccharides, have a significant effect on the gastrointestinal tract, and they are resistant to acid conditions in the human stomach as a natural source of prebiotics. Fructooligosaccharides are partially resistant to human salivary α-amylase, and they also stimulate the growth of Lactobacilli and Bifidobacteria (Khuituan et al., 2019; Wichienchot et al., 2010). Increasing the number and activities of beneficial bacteria in the human gut microbiota compared to pathogens has a great importance for human health, and at this point, the positive results of prebiotics on the microbiota draw attention. According to a study; It has been shown that free oligosaccharides isolated from pitaya have quite a prebiotic effect (Dasaesamoh et al., 2016). In addition to their free forms, oligosaccharides can also be found bound to proteins by glycosidic bonds. The biological properties of these bound oligosaccharides have not been adequately clarified due to the inadequacy of the techniques used to separate these glycans from glycoproteins.

It is claimed that many oligosaccharides have a prebiotic effect, but not every oligosaccharide has a prebiotic effect and must meet some criteria (Khalili et al., 2014). First of all, for oligosaccharides to have prebiotic properties, they must not be digested by host enzymes in the stomach and small intestine (Casci et al., 2006). Secondly, these substances must undergo fermentation in the digestive tract (Gibson and Rastall, 2012). Finally, prebiotics should be selective for beneficial bacteria in the gut, such as *Bifidobacteria* and

*Lactobacilli*, and affect the metabolic activity of these bacteria. Thus, as a result of fermentation by bacteria using prebiotics, it should have a beneficial effect on the host.

### 1.2. Glycosylation and Importance of *N*-Linked Glycans

Glycans may exist in the form of free oligosaccharides or glycoconjugates. The process by which oligosaccharides bind to proteins to form glycoproteins is called glycosylation. Glycosylation primarily affects the development and function of some regions of the brain and the gastrointestinal system, as well as the functions of the hepatic, visual and immune systems, which indicates the importance of glycosylation in the body (Freeze et al., 2015). More than 50% of eukaryotic proteins are in glycoprotein structure (Strasser, 2016). Glycoproteins, which are a glycoconjugate, formed by the covalent attachment of one or more glycan-carrying proteins to a polypeptide backbone, usually *N*- or *O*-linked (Varki et al., 2017).

*N*-acetylglucosamine (HexNAc) attaches *N*-linked glycans to the amino group of asparagine (Asn) residues of proteins in a specified amino acid sequence Asn-X-Ser/Thr or Asn-X-Cys (cysteine) (where X may represent any amino acid other than proline) (Lafite and Daniellou, 2012). *N*-linked glycans comprise a single core with two N-acetylglucosamine (GlcNAc) residues subsequently followed by three mannose groups. Continued glycosylation defines the types of N-linked glycans, which can be categorized into three major classes based on their composition: complex, high mannose (HM), and hybrid. (Varki and Lowe, 2009).

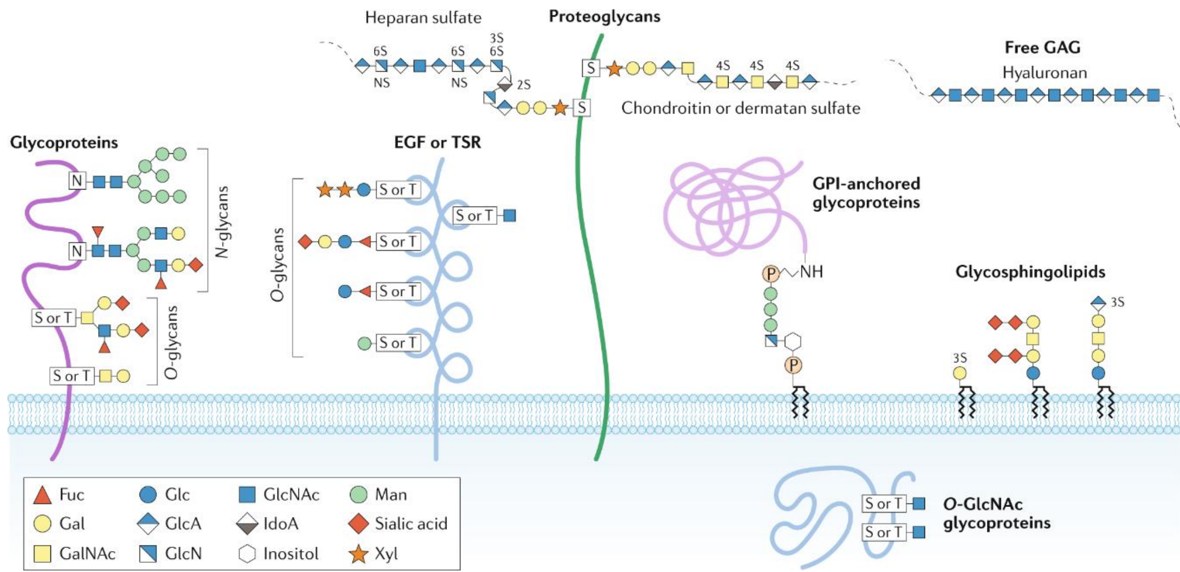



Figure 1. Main glycosylation types found in human (Reily et al., 2019)

*O*-linked glycans are attached to the –OH group on the side chains of serine (Ser) or threonine (Thr) amino acids in the polypeptide chain through *N*-acetyl galactosamine (GalNAc) in their structures and can form eight different nuclei (Varki et al., 2017). All other glycans are synthesized by glycosyl transferase and glycosidase enzymes adding different glycan monomers to these nuclei (Rini and Esko, 2015). Excessive branching, especially in structures of *N*-linked glycans, is usually encountered in cancer cells, and the increase in these branches causes cancer to progress (Varki et al., 2015). Therefore, studies targeting *N*-linked glycans in cancer treatments are likely to provide appropriate responses for future treatments. Additionally, important proteins involved in eukaryotic secretory pathways are mostly regulated by *N*-linked glycans. It is also known that *N*-linked glycans regulate glycoprotein folding by using glycoprotein balance (Helenius and Aebi, 2001). Complex *N*-linked glycans are important in their interactions, such as the maintenance of growth factors and the ability of cell surface cytokine receptors to convert $\beta$ growth factor of glycan-linked proteins such as galectin and cytokines (Stanley P, Taniguchi N, 2017). Studies on glycoprotein balance show promise in developing drugs that can be a solution to various diseases in the near future.

One of the main factors that influence the nature and physiology of the microbiota is the intake of glycans into the gut, primarily through diet and host mucosal secretions. (Koropatkin et al., 2012). Humans consume dietary glycans from dozens of different plants and animals, many of are incapable of being broken down by enzymes encoded in the genome humans. These indigestible glycans are converted into short-chain fatty acids (SCFAs) via microbial fermentation, which serves as nutrition for other intestinal epithelial cells (Chambers et al., 2018). Intestinal microorganisms thus play a crucial probiotic role by supporting humans to use indigestible nutrients. Each of the microorganisms in the body prefer to consume different glycans. Therefore, consuming these nutrients selectively may influence the proliferation of microbial populations found in GI system, indicating a noninvasive strategy that dietary glycans can directly affect humans' intestinal balance (Koropatkin et al., 2012). Glycans can be consumed, expressed and released endogenously by host cells, or generated by microbes. Glycans can be consumed, expressed, and released endogenously by host cells, or synthesized by microbes. All of these procedures indicate that they are suitable for the gut microbiome and that glycans play a prominent role in host-diet-microbe interaction. The most notable change in glycans as a dietary source is the transition from a dairy-based neonatal diet to an adult diet. This transition significantly impacts immunological development because it affects the diversity of the gut microbiome, microbe gene expression, and host cell gene expression, ultimately impacting mucin composition. (McKeen et al., 2019). Discovering the glycan-mediated interactions that take place during this shift may lead to dietary guidelines to enhance gut and immunological development.

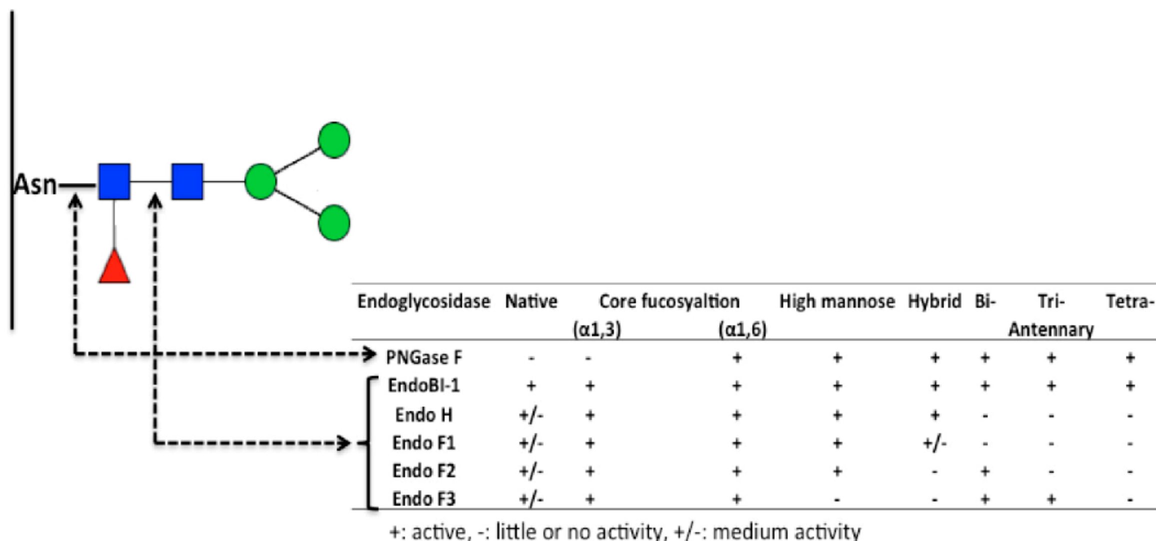
*N*-glycans have important roles in several cellular pathways crucial to health and disease. Their relevance in cell mobility and adhesion, activation of receptors, structural changes in protein including degradation and folding, calnexin-calreticulin cycle, immune signaling, defense against microbial and viral attacks, as well as modulation and development of infant gut microbiome has been proven by research (Marcobal et al., 2010). Similar to monosaccharide construction of N-glycans, and binding types to Human Milk Oligosaccharides (HMOs) in many aspects, have also been proposed to have essential bifidogenic activity either through direct growth impulse or through antimicrobial effects. Recent studies have demonstrated that release of *N*-linked glycans via new glycosidase Endo-β-*N*-acetylglucosaminidase (EndoBI-1), from bovine milk glycoproteins, stimulate the growth and development of *Bifidobacterium infantis* selectively. *In-vivo* studies recently

indicate that N-glycans attached to lactoferrin and immunoglobulins stimulate the growth of *B. infantis*. This study also revealed that EndoBI-1 from *B. infantis* with unique function and structural specificity for the release of N-linked glycans found in milk glycoproteins is active under conditions encountered *in-vivo* (Karav et al., 2019). Based on these *in-vivo* and *in-vitro* studies, it is evident that different glycan types effects on various way to shape the gut microflora.

### 1.3. Glycan Release from Glycoprotein Sources by the Human Microbiome Derived Enzyme Endo-*β*-*N*-acetylglucosaminidase

Removal of glycans from glycoproteins is an essential step to study glycans. Chemical techniques are widely used in glycobiology to deglycosylate glycoproteins since these methods are quick and simple to use, inexpensive, and have a high level of substrate specificity (Li et al., 2019). The chemical deglycosylation processes hydrazination and β-elimination are frequently used in glycan research. *β*-elimination is based on the cleavage of glycans by exposing the glycoproteins to alkaline conditions. This process may result with degradation of released glycans and sample losses due to the high salt concentration in the cleaning stage (Turyan et al., 2014). The disadvantage of this approach is that it hampers the control of glycan release as they have only a single labeling group that is converted to alditols by the reducing agent and prevents binding of a fluorophore or chromophore. (Turyan et al., 2014). The hydrolysis reaction that occurs after the addition of anhydrous hydrazine to the glycoprotein is the basis for hydrazine treatment. This approach is widely applied because it enables controlled isolation of *N*- and *O*-glycans by altering reaction parameters including temperature. Peptidyl-*N*-glycosidases (PNGases) are the enzymes that widely used to release glycans from glycoproteins. (Altmann et al., 1995). A PNGase cleaves asparagine-linked glycans in order to hydrolyze the amide side chain. Commercial PNGases have ability to release *N*-glycans in every charge and size. However, in the presence of fucose, since it linked to  linked to *N*-acetylglucosamine, PNGases cannot be active to cleave the glycans (Tretter et al., 1991). The shape of the glycoprotein is an additional obstacle to PNGase activity on *N*-glycans. Activity of PNGases necessitates denaturing glycoproteins with high heat and detergent to improve enzyme accessibility to glycans. Nevertheless, However, these extreme conditions may cause the released glycan and remaining polypeptide structures to

be disrupted, which is directly related to the biological activity of these molecules. PNGase F is less active on the natural form of proteins than endoglycosidases F1, F2, and F3, while their activity on multiple-antennary glycans is extremely low (Trimble and Tarentino, 1991).




| Endoglycosidase | Native | Core fucosyaltion (α1,3) | Core fucosyaltion (α1,6) | High mannose | Hybrid | Bi- Antennary | Tri- Antennary | Tetra- Antennary |
|---|---|---|---|---|---|---|---|---|
| PNGase F | - | - | + | + | + | + | + | + |
| EndoBI-1 | + | + | + | + | + | + | + | + |
| Endo H | +/- | + | + | + | + | - | - | - |
| Endo F1 | +/- | + | + | + | +/- | - | - | - |
| Endo F2 | +/- | + | + | + | - | + | - | - |
| Endo F3 | +/- | + | + | - | - | + | + | - |

+: active, -: little or no activity, +/-: medium activity

Figure 2. Endoglycosidases and their specificity on *N*-glycans (Karav et al., 2017).

A novel enzyme Endo-*β*-*N*-acetylglucosaminidase (EndoBI-1), naturally released from *Bifidobacterium longum subsp. infantis* ATCC 15697, has ability on successfully cleavage of the *N*-*N'*-diacetylchitobiose moiety present in the complex, high mannose and hybrid *N*-glycan cores (Karav et al., 2015). Fucosylation of the N-glycan core has no effect on EndoBI-1 activity. In addition, changing environmental conditions such as pH, temperature and reaction time has no effect on enzyme activity. It has also been shown to be heat resistant, allowing it to be used in 95°C heat treatments and, can also release glycans without substrate denaturation compared to the current *N*-acetylglucosaminidases including PNGases (Karav et al., 2015; Parc et al., 2015). In this thesis, EndoBI-1 enzyme used to release *N*-glycans from different pitaya glycoproteins.

### 1.4. *In-Vitro* Digestion Models Developed For Bioactive Food Components

Understanding the effects of nutrition and diet on human health, especially the digestive system, is an increased interest for food and health industries. Nowadays, a

growing number of individuals are concerned about living healthy lives, one of which is proper dietary consumption. They recognize the need of consuming healthy nutrients that rich in bioactive components. Studying on digestive system are getting harder due to the digestive system has relationships with many other human physiological systems. In order to make studies easier and faster, researchers have found different *in-vitro* digestion models (Marcano et al., 2015; Minekus et al., 2014). *In-vivo* studies have some diasadvantages such as technical and etnical diffuculties, cost and biological, physiological differences between individuals. New *in-vitro* models are better alternatives for these complex studies. DeBaun and Connors were the first researchers to use the *in-vitro* digestion model (DeBaun and Connors, 1954). *In-vitro* digestion systems mimic the human physiological conditions of digestion oral, gastric and small intestine parts *in vivo*. *In-vitro* digestion models vary based on chemical content for digestion part of system. By means of these technique understanding interactions, changes and bioaccessibility between different molecules and digestive system is much easier. Currently *in-vitro* digestion models role on new applications such as nutrition, pharmacology and food chemistry fields.

### 1.4.1. Static *In-vitro* Digestion

Digestion of foods is a quite complex process and several different digestion models have been created to understand physiological changes and bioaccessibility of nutrients after digested by human body (Xavier and Mariutti, 2021). These models aim to predict the behavior of food components taken into the human body as closely as possible to the actual digestion model conditions.

Static in vitro digestion models are the most functional and simplest methods for mimicking *in-vivo* food digestion. Static models have been used to search for several uncovered scientific questions including diet associated disorders and their mechanisms, and bioaccessibility of a wide range of nutritional components (Xavier and Mariutti, 2021). Even though various models are presented to the literature, there are significant variations among digestion parameters. Each model created in certain circumstances for understanding significant effect of specific compounds. This situation creates an obstructive condition for compare results between research groups and make inferences. While changing some of

these factors might not have much of an effect on the matrix release or digestibility of certain compounds, it might have a considerable effect on other constituents.

Recently, people have been a surge in curiosity about the digestion process and uncovering chemical and biological mechanisms including the possible impact on the body after nutrients consumed. Revealing the fate of nutrients and vital bioactive constituents are critical for designing new products with specific functions or technologies that boost the functional potential of everyday meals and more nutritive dietary foods. This comprehension required for metabolic and bioactivity studies, which give evidence of the positive or harmful effects of these substances on human health (Mackie et al., 2020).

Due to their simplicity, static models have been used in several years for food and drug industries. These models are easy to apply with a fixed ratio of sample, enzymes and electrolytes as well as a constant pH for each step of digestion. It has been demonstrated that static in vitro digestion models are quite effective to estimate how digestion will behave in vivo. Additionally, it provided experimental guidelines to enhance the comparability, reproducibility and uniformity of in-vitro digestion studies (Egger et al., 2016). Besides, static digestion methods have not been able to represent the intricate dynamic process in host physiology as well as transit between digestive phases.

### 1.4.2. Semi-Dynamic *In-vitro* Digestion

Semi-dynamic approaches have recently been created and enhanced to bridge the gap between dynamic and static in-vitro digestion methods. Semi-dynamic approaches aim to simulate the kinetics of the digestive system, particularly in the gastric phase, including pH changes, enzyme secretion, and stomach emptying. Semi-dynamic approaches are therefore appropriate for studying structural changes in food digestion, such as the influence of the food matrix on decomposition and delivery of nutrients (Xavier and Mariutti, 2021).

Fundamental advantage of this semi-dynamic models is mimicking of the ephemeral nature of gastric conditions, progressive acidification and gastric emptying. This enables for the assessment of the alterations in the structure of food and dissociation. For example, semi-dynamic system enables visual recording of the alterations throughout gastric digestion at

the macroscopic grade (Mulet-Cabero et al., 2020). Additionally, this systems enable to in-situ profiling dynamic pH at every point of gastric emptying. Several physiological and chemical changeable might be appraised in the aliquots to monitor the digestion kinetics of nutrients (Mulet-Cabero et al., 2020).

Consequently, the semi-dynamic gastric models are promising approaches to designate degree of nutrient bioaccessibility that provide more reliable course than other methods (Markussen et al., 2021). The semi-dynamic systems are available to use with variable capacity of amount and convenient to use without a large necessity of high cost enzymes. Simplicity in use and low necessity of specialized equipment makes this method advantageous and practical for diversified foods and usefully implemented in researches all over the world.

### 1.4.3. Dynamic *In-vitro* Digestion

The human digestive system is a dynamic and complex environment that is affected through many internal and external variables in its natural process. Simulation of human digestion as a dynamic system requires several variables to recreate the biological environment. Among these factors geometry of gastrointestinal compartments, physical forces, and biochemical environment, including the release of enzymes, hormones, salts, lipids, and many other components to regulate the digestion process of a host directly and indirectly.

Scientists have developed several dynamic in-vitro digestion models to decode the fate of consumed foods or xenobiotics by simulating dynamic conditions of the human body. Since many of the considerations and variables required to design an in-vitro model, a wide range of those models are available in the literature, created according to investigated sample or under researched effector through a controlled manner.

Comparing the static models, dynamic models can mimic the in vivo digestion conditions by changing conditions of different compartments. Controlled parameters for the digesting processes sorted as digestive fluid, variable enzymes involved in digestion, pH, continuation period, peristalsis, absorption, and emptying were set by the dynamic in vitro

model (Dupont et al., 2019). By this way dynamic models enables include gradually a variety of digestive secretions into the model's compartments. Including this secretions can have a constant rate or can follow a scheduled change in accordance to other variables. In dynamic models pH is frequently followed based on real-time data used to manage the proportion of hydrochloric acid addition that provides the acidification of the consumed product within the gastric conditions. In this model, pH of gastric cyhme is neutralized by regulated addition of sodium bicarbonate and other necessary enzymes. However, in static digestion methods are not capable to provide mechanical forces, pH adjustments and secretion of enzymes. Additionally in static models, complex digestive physiology is not taken into account in terms of geometry and nature of the system.

For this reasons, dynamic models have better accuracy rate to mimic in vivo conditions and gives closer results in terms of understanding digested product and their effect on human body. However, necessity of devices and expensive materials makes dynamic models impractical and pricey. Therefore, according the digested sample type and research aim scientist mostly prefer static methods to obtain results easily, and practically.

### 1.5. Prebiotics and Probiotics as Strong Health Promoters

The term "prebiotics" encompasses food components that are resistant to digestion by the host organism, yet possess the remarkable ability to selectively promote the growth and function of specific bacteria within the colon, thereby contributing to enhanced host health. Prebiotics, characterized by their nondigestible nature, serve as fermentable substrates that actively stimulate the growth and metabolic activity of beneficial bacteria that already inhabit the colon of the host organism. Despite their resistance to digestion, prebiotics plays a pivotal role as fermentable substances, eliciting a range of physiological responses that support the proliferation and functionality of beneficial bacterial populations within the host colon. These prebiotic compounds act as substrates for the fermentative metabolism of resident microorganisms.

While prebiotics primarily interact with the colon microflora, they are also involved in many other processes through the modulation functionality of immunity in other systems, including mucosa, oral cavity, urogenital tract, and the small intestine. Prebiotics are found

in various sources, including unrefined wheat, soybeans, breast milk etc., but the great majority is dietary fibers such as oligosaccharides. (Durazzo et al., 2020).

Fruit oligosaccharides are a type of prebiotic source that are regularly consumed by human populations. Since these type of carbohydrates cannot be digested by human origin enzymes, they are able to reach to the colon as an intact form to involve microbial digestion process as a prebiotic source. Thousands of fruits found all over the world have different level of carbohydrate and protein content. That difference provides the diversity of glycans and dependently affects prebiotic activity when consumed.

Latest studies on probiotics comprised clinical trials involving healthy adults. In general, commercial probiotics (either one strain or several) were the most popular strategy among them. The observed outcomes suggest that consuming probiotics may favor the gut microbiota by boosting the amounts of *Bifidobacteria* or changing *Lactobacilli* subpopulations. Furthermore, probiotics were linked to promoting interactions between key microbiota constituents and the host epithelium, boosting immunity, and improving bowel movements (Markowiak and Ślizewska, 2017).

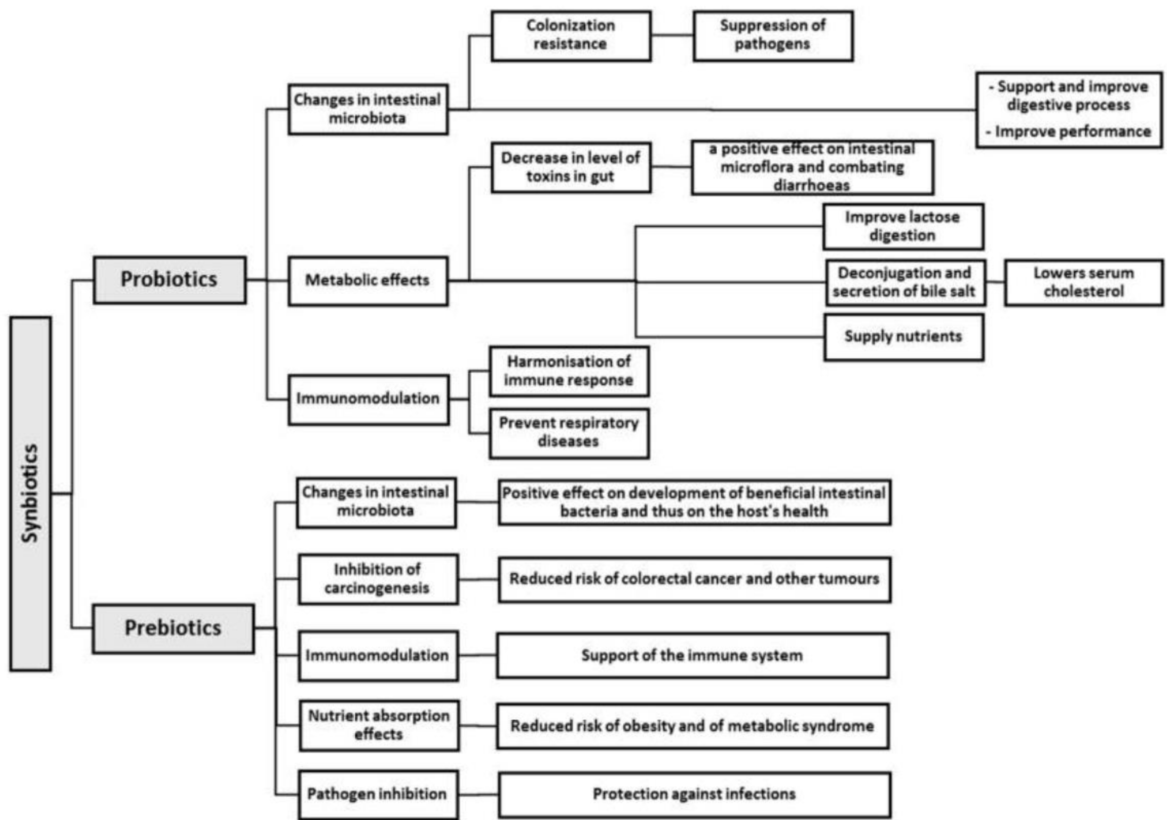



Figure 3. Effects of probiotics and prebiotics on health (Markowiak and Ślizewska, 2017)

# CHAPTER 2
# PREVIOUS STUDIES

A novel in-vitro digestion assay used to understand behaviour ofpitaya glycoproteins and an analysis carried out to determine prebiotic potential of *N*-glycans obtained from pitaya within the scope of this thesis. There is a lack of information and analysis available in the literature about pitaya glycans and their prebiotic effect. The following section summarizes the research done on this topic.

## 2.1. Previous Studies

Prebiotics and probiotics are two vital components of a healthy diet that can have a substantial impact on health support and disease prevention. Recent studies have shown prebiotics feed the good bacteria in our gut, while probiotics are living microorganisms that can provide a variety of health benefits when ingested in adequate amounts (Karav et al., 2019). Studies have prove supplying necessary nutrients for probiotics provides a balanced and healthy environment to live in. Prebiotics and probiotics cooperate to support a thriving microbiome in the gut and improve quality of life. There are very limited studies about prebiotic activity of pitaya and pitaya glycans that need to be further analyzed.

Animal studies about the oligosacchrarides of pitaya have found a variety of potential digestive health benefits via boosting fecal production and intestinal motility, and stimulating laxative (Khuituan et al., 2019). As a dietary supplement, consuming pitaya oligosaccharides may boost gut health and repair gastrointestinal motility problems. In other words, consuming pitaya oligosaccharides may aid in the stimulation of 'easy-to-pass' bowel movements. (Wichienchot et al., 2010). Although there are studies on the oligosaccharide content of pitaya, its *N*-linked glycan content is still unknown. In order to characterize glycans, examine their effects in different studies and understand their functions, it is necessary to separate them from the proteins they are attached to.

Understanding the fate of food products throughout body after digestion searched by several in-vitro digestion methods. The most enhanced static system known as INFOGEST that developed by several researcher to work on food components (Mulet-Cabero et al., 2020). However this system has limitations as it lacks microbial enzymes. Consequently, the outputs of study, enlightened the prebiotic activity of dragon fruit *N*-glycan profile and, revealed the fate of pitaya glycoproteins more specifically through the in vitro digestion method contain microbial enzymes and prebiotic activity studies of bioactive *N*-glycans.

# CHAPTER 3
# MATERIAL AND METHOD

## 3.1 . Materials

This subsection will list all of the materials required to complete the thesis experiments, comprising chemicals, substrates, and laboratory equipment.

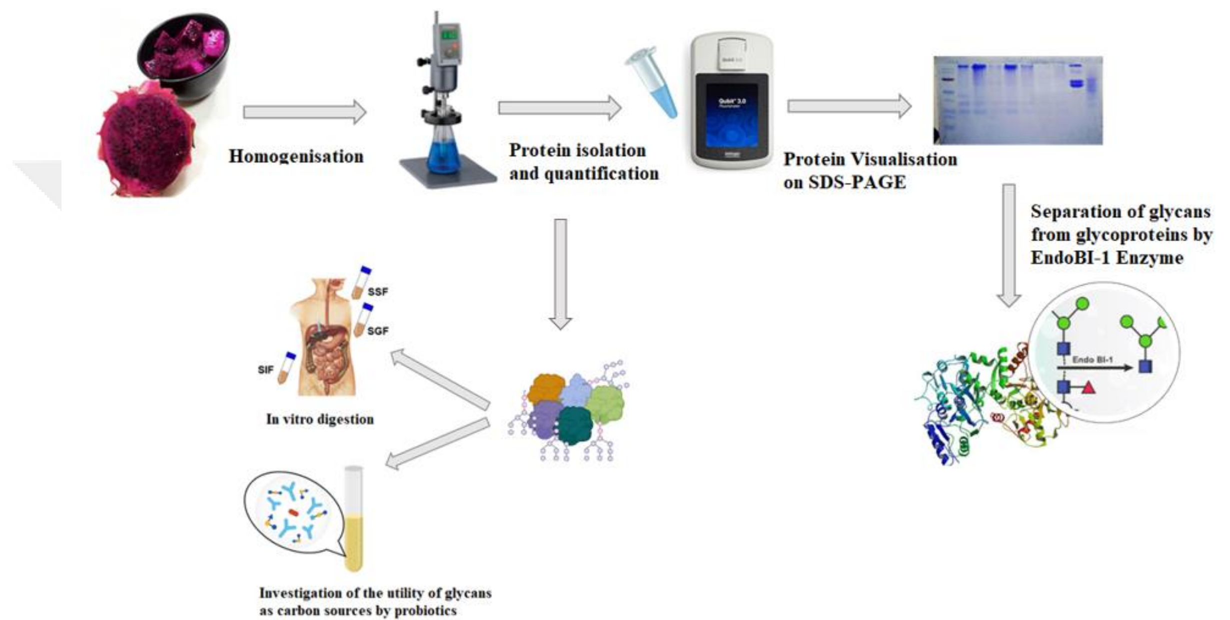



Figure 4. General workflow diagram for methodology

### 3.1.1. Substrates

The enzymatic deglycosylation studies were performed using pitaya fruits obtained from Thailand and the local market. In addition, PNGase F enzyme (P0704S) was used to compare and confirm deglycosylation via mass spectrometry.

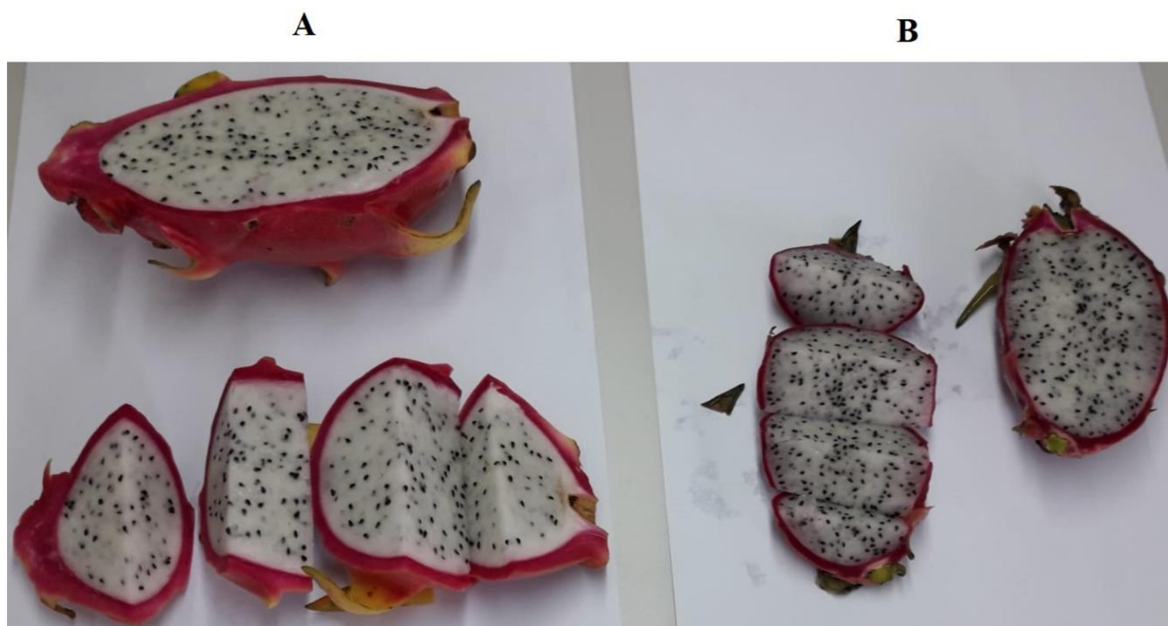



Figure 5. Samples belong to dragon fruit. A and B represent fruits originating from Thailand and Türkiye respectively.

### 3.1.2 Chemicals, Kits and Required Items

All laboratory equipment and chemicals used in this thesis is given below (Table 1).

Table 1

List of materials, suppliers' catalog number and usage information

| Name of Materials | Catalog Number | Information |
|---|---|---|
| 10-kDa-cut-off centrifugal filter | UFC9010 | Enzyme concentration |
| 10X Running Buffer (Tris-Glycine SDS) | TGS10 | SDS-PAGE visualisation |
| Acetic acid | 137130 | SDS-PAGE visualisation |
| Ammonium Persulfate | 17874 | SDS-PAGE visualisation |
| Coomassie Brilliant Blue R 250 | 0472-25G | SDS-PAGE visualisation |
| EndoBI-1 | | |
| Ethanol | 920.026.2500 | Protein isolation |
| Glycobuffer 2, | P0704S | Buffer for glycan release |

| | | |
|---|---|---|
| Glycoprotein Denaturing Buffer, NP-40 | | |
| Imidazole | 56750 | Cell lysis |
| Isopropanol | 961.023.2500 | SDS-PAGE visualisation |
| Laemmli Sample Buffer (2X) | LSB-2x | SDS-PAGE visualisation |
| Methanol | 106012 | SDS-PAGE visualisation |
| Phenol | P1037 | Phenol sulfuric acid assay |
| PNGase F | P0704S | Release of glycans from glycoproteins |
| Qubit™ Protein Assay Kit | Q33211 | Determination of protein concentration |
| RPMI 1640 (no glucose) | 11879020 | Bacterial growth |
| Sodium Chloride | 31434-5Kg-R | Cell lysis |
| Sodium dodecyl sulfate (SDS) | SDS001.500 | SDS-PAGE visualisation |
| Sodium Phosphate Dibasic Dihydrate | 04272-1Kg | Buffer for cell lysis |
| Sodium Phosphate Monobasic Anhydrous | 0571-1Kg | Cell lysis buffer |
| Sulfuric acid (95-98%) | 112080 | Phenol sulfuric acid assay |
| SureCast™ Acrylamide Solution (40%) | HC2040 | SDS-PAGE visualisation |
| SureCast™ TEMED | HC2006 | SDS-PAGE visualisation |
| Tris | TRS001.1 | Cell lysis |
| Tris base | TRS001.1 | SDS-PAGE visualisation |

### 3.1.3. Laboratory Equipments

In the course of conducting research for this thesis, a range of laboratory equipment was used. All equipment was accessed through the facilities of Canakkale Onsekiz Mart University (COMU) Molecular Biology and Genetics Department research laboratory. The following is a comprehensive list of all equipment used (Table 2).

Table 2

List of Laboratory Equipment used for the thesis

| Device name | Brand name |
|---|---|
| -20˚C freezer | Arçelik |
| Analytical balance | Shimadzu |
| Anaerocult | Merck, Germany |
| Autoclave | NÜVE |
| Bruker rapifleX™ MALDI Tissuetyper™ | Bruker Daltonik GmbH, Bremen, Germany |
| Centrifuge | Beckman Allegra X-15R |
| Cooling centrifuge | Hettich Mikro 200 R |
| Dry bath | Benchmark Scientific BSH1002-E |
| Ice generator | Izmak |
| Incubator | Indem Nüve EN 400 |
| Orbital shaker | STUART |
| pH meter | IsoLab |
| Power supply | Bio Rad |
| Pure water system | Millipore |
| Qubit™ 3 Fluorometer | Invitrogen |
| Speedvac evaporator | Labconco |
| Thermal shaking incubator | INOVIA |
| Thermometer-Portable | IsoLab |
| Vibra Cell/Sonicator | Sonics |
| Vortex | Vortex Genie 2 |

### 3.2. Method

### 3.2.1. Preparation of Pitaya Samples and Protein Isolation

In this thesis, locally produced and imported pitaya fruits, were used during this study. The fruits included in the study were supplied in ripe form from two different origin; Thailand an Türkiye. The pitaya samples was cleaned under running water and peeled, then flesh of the fruit was chopped into small pieces. For protein isolation, firstly, the samples were homogenized using a homogenizer (Heidolph Silent Crusher) after being dissolved with distilled water with a of 1:4 ratio. For further homogenization of the obtained samples, a secondary homogenization process (Vibra Cell/Sonicator) were applied. Then, a 1:1 mixture of protease enzyme inhibitor and lysis buffer solution (1:100 v/v) was involved to the samples and incubated for 1 hour. After incubation, the samples were centrifuged under conditions of 10 min, 4000 rpm, 4°C. The proteins were precipitated by adding 1:4 cold ethanol to the supernatant obtained after centrifugation (1 hour -20°C incubation). Then, it was centrifuged again along 10 min., at 4000 rpm, and 4°C. The pellet part (protein) dissolved in pure water and stored at -20°C for further steps (Karav et al., 2017).

Figure 6. Pitaya Protein Isolation Process

### 3.2.2. Visualization of Pitaya Proteins

SDS-PAGE gel electrophoresis (Invitrogen Mini Gel Tank) was used to display the protein profile and molecular weights of Pitaya samples. SeeBlue™ Pre-stained Protein Standard (Invitrogen) was used as the marker. Protein samples were mixed with Laemmli Sample Buffer (1:1) incubated for 10 min at 95˚C. Following the incubation incubation, 40 µL from each sample mixture was loaded to a gel prepared according to the Laemmli procedure. Jel prepared as 4% stacking gel (40% Acrylamide/ Bisacrylamide, 1 M Tris pH 6.8, 10% SDS, 10% Ammonium persulfate, TEMED, distilled water) and 12% resolving gel (40 % Acrylamide/Bisacrylamide, 10% SDS, 1.5 M Tris pH 8.8, 10% Ammonium persulfate, TEMED, distilled water). The loaded gel run for 60 min at 200 V with Novex™ Tris-Glycine SDS Running Buffer (1X). Samples were incubated with Coomassie Brilliant Blue for 20 min at 50 rpm. Then, proteins were destained with a destaining solution (50% $dH_2O$, 40% Methanol, 10% Glacial Acetic Acid) for an hour (Laemmli, 1970).

### 3.2.3. Protein Quantification By Qubit Fluorometric Assay

The protein concentration of the pitaya samples was quantified with a Qubit 3.0 fluorometer (Thermo Fischer Inc, CA USA). After calibrating the device with appropriate standards, a working solution was prepared using Qubit™ protein buffer and Qubit™ protein reagent, then 198 µl of this solution was taken and 2 µl of sample was added and incubated in a room temperature for 15 min. (Table 3). Measurements were taken following the incubation process and sample protein concentrations were measured by multiplying the result obtained by the dilution factor.

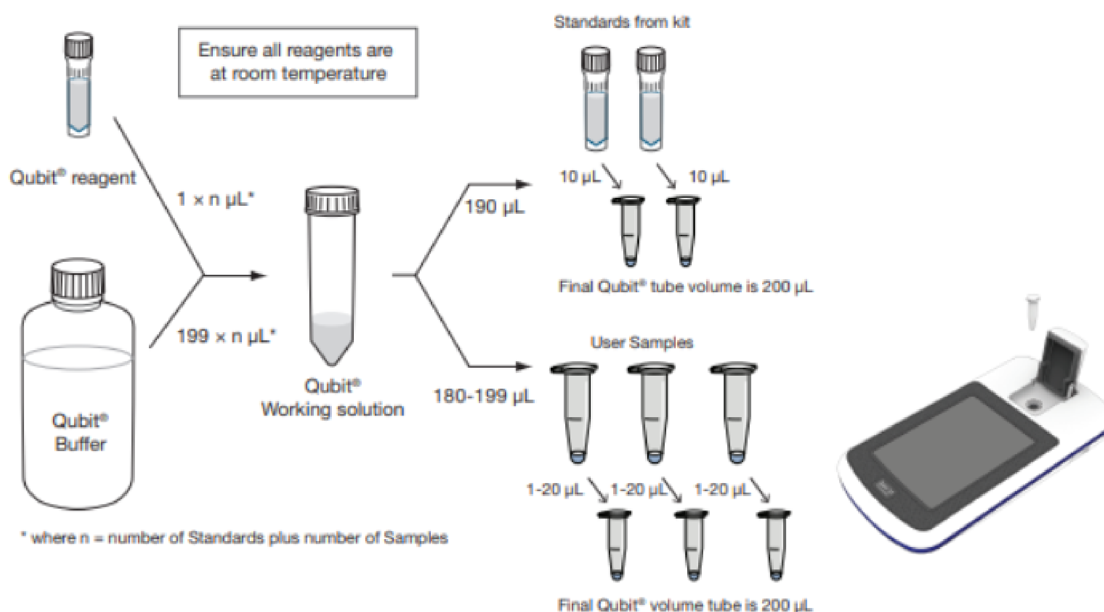



Figure 7. Working Principle of Qubit Fluorometer (Thermo Fisher Scientific Inc., 2014).

Table 3

Qubit 3.0 Fluorometer Standard and Sample Solution Volumes

| | Standard Tube | Local Pitaya | Imported Pitaya |
|---|---|---|---|
| **Working Solution Volume** | 190 µl | 198 µl | 198 µl |
| **Standard Volume** | 10 µl | - | - |
| **Sample Volume** | - | 2 µl | 2 µl |
| **Total Volume** | 200 µl | 200 µl | 200 µl |

### 3.2.4. Release of *N*-Linked Glycans from Pitaya Glycoproteins

Separation of *N*-linked glycans from Pitaya proteins was performed with EndoBI-1 and commercial PNGase-F enzymes. The enzymatic reaction for EndoBI-1 was carried out under optimum conditions (pH 5, 37°C and 0.02 M $Na_2HPO_4$ buffer) determined in previous

studies (Parc et al., 2015). After completion of glycan separation, deglycolyzed proteins were precipitated with cold ethanol (4:1 ratio). The supernatant part including the released glycans was dissolved in 100 µL of $dH_2O$, flowing the drying with a vacuum evaporator.

Additionally, pitaya glycans released by using a commercial PNGase-F enzyme kit to verify novel glycan structures in mass spectrometry.

### 3.2.5. Detection of Glycan Release via Colorimetric Method

Glycan release was confirmed by the Phenol Sulfuric Acid method (Masuko et al., 2005). The stock glucose was prepared by dissolving 1 mg glucose with 1 mL distilled water so that 1 mg/mL. By serial dilution, 100, 200, 400, 600, 800, 1000 µg/mL glucose standards were diluted to a final volume of 0.5 mL. 25 µL from of each standard was added into the 96-well plate. First, 25 µL of 5% (w/v) phenol solution was added on the standards, then mixed thoroughly by pipetting. Consequently, 125 µL of concentrated sulfuric acid was added and mixed quickly by pipetting. Pitaya glycans samples were prepared in the same way as the standards, using 25 µL of sample, 25 µL of 5% (w/v) phenol solution and concentrated sulfuric acid. After this process, the prepared samples were incubated at 35 rpm for 30 min in the dark. Later incubation, OD value was measured at 490 nm. All samples were prepared in duplicate.

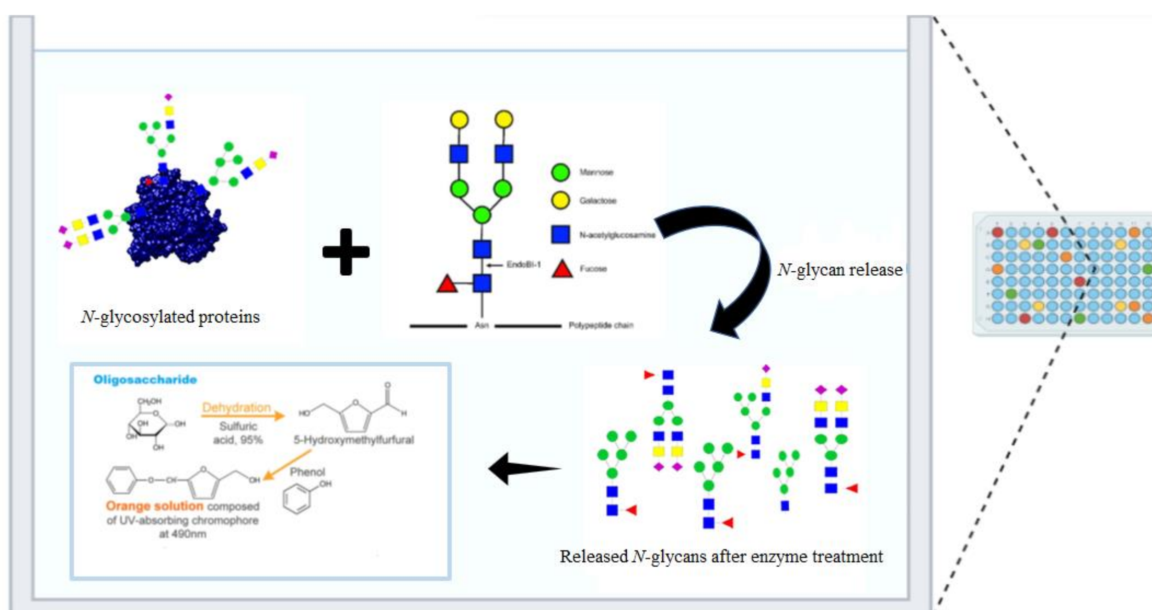


Figure 8. Glycan Release Principle of Pitaya Glycoproteins and Detection via Colorimetric Method

### 3.2.6. *N*-Glycan Characterization by Mass Spectrometry

MALDI-TOF-MS was used for the analysis of *N*-glycans. *N*-glycans were labeled with 2-aminobenzoic acid (2-AA). Labeling process was performed according to Ruhaak methods (Ruhaak et al., 2008). 50 µL of glycan solution was mixed with 25 µL of 2-aminobenzoic acid and 25 µL of 2-picoline borane, then this mixture was incubated at 65°C for 2 h. A pipette tip containing cotton was used for the purification of glycans (Selman et al., 2011). Accordingly, cotton fibers were placed in a pipette tip of 100 µL. Subsequently, the sample exposed to ethyl esterification was used as the loading solution. The prepared pipette tip was washed sequentially with 100 µL of $dH_2O$ and 100 µL of 85% acetonitrile. The prepared loading solution was passed through the pipette tip containing cotton at least 20 times. To remove salt and other solvent residues, the pipette tip was rinsed three times with 85/14/1, v/v/v, acetonitrile/water/trifluoroacetic acid solution and three times with 85% acetonitrile to. The elution process was performed with 10 µL of $dH_2O$. For MALDI-TOF-MS analysis, 1 µL of elution solution was dripped onto MTP 384 Anchor Plate and dried. Steps that follow, 1 µL of 5 mg Super DHB matrix solution (w/w, 9/1, 2-5 dihydroxy benzoic acid / 2-hydroxy-5-methoxy benzoic) prepared in 1/1, (v/v) acetonitrile/1 mM NaOH was added to this spot. After crystallization occurs, analysis with MALDI mass spectrometry was performed with a Bruker rapifleX MALDI Tissuetyper 3D 10kHz 355 nm Nd YAG laser. Analysis was conducted by collecting at least 10000 laser shots in reflectron mode in positive ionization. Before each analysis, Bruker rapifleX MALDI TissuetyperTM mass spectrometry calibration was performed with Ovalbumin Tryptic digest. By applying 25 kV acceleration voltage, mass spectra were obtained between 1000-4000 mass range. Analysis of 2-AA labeled *N*-glycan structures were assessed in negative ionization mode using the method described above using the a 5 mg/mL DHB (50/50, ACN/$H_2O$) matrix solution. Spectra with a frequency of 5000 Hz and a mass range of 1000-4000 Da were obtained (Kayili et. al, 2023).

### 3.2.7. Mass Spectrometry Data Analysis

The composition of the collected MS spectra was examined using GlycoQuest software after they were transferred to Protein ScapeV4. In consequence of analysis identified *N*-glycans were determined.

### 3.2.8. *In-vitro* Digestibility Test of Potential Prebiotic Glycans

Glycoproteins obtained from pitaya samples were tested via *In-vitro* Digestion method developed by thesis study of Kaplan M., 2022.

Pitaya protein samples (2 g) was filtered using 10 kDa Amicon tube to remove contaminants including free oligosaccharides was used as a glycoprotein source that includes a high concentration of glycans. All digestion solutions including simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared (table of content given in Appendix 2). All digestion solutions and enzymes were preincubated at 37°C before the experiment. 1 mL of the sample was removed after each phase and kept at -20°C until the next usage. In general, in the oral phase, pitaya proteins which does not contain free oligosaccharides, was added to a 3.5 mL SSF stock solution. Then, human salivary a-amylase (EC 3.2.1.1, 15000 U mL-1) and 25 µL, 0.3 M $CaCI_2$ were added to the solution. Finally, 975 µL distilled water was added to the mixture and mixed well. The incubation time for the oral phase took 2 min. at 37°C shaking by hand. In the gastric phase, the final ratio of food to SGF solution was at 50:50 (v/v) after adding other components. A 10 mL liquid sample was added to a 7.5 mL SGF solution and then 1.6 mL pepsin (from porcine gastric mucosa 3200- 4500 U mg-1) was mixed with the mixture. 5 µL, 0.3 M $CaCI_2$, and 1 M HCI for keeping pH at 3.0 and 0.695 µL distilled water were added to the final mixture. The incubation time for the gastric phase was 2 hours at 37°C, 100 rpm. In the intestinal phase, the final ratio of gastric chyme to SIF stock solution was at 50:50 (v/v) after adding other chemicals and distilled water. 1 M NaOH was required to adjust pH at 7.20 mL of gastric chyme from the previous phase was mixed with 11 mL of SIF solution. 5 mL pancreatin solution (from porcine pancreas, 800 U mL-1), 2.5 mL, 160 mM fresh bile, 40 µL, and 0.3 M $CaCI_2$ were added to the mixture. Finally, 0.15 mL of 1 M NaOH was added to adjust pH at 7.0 and 1.31 mL of distilled water was mixed with the final solution.

The incubation time for intestinal digestion took 2 hours at 37°C, 100 rpm. During the experiment, 2 µL of each recombinant enzyme was integrated into appropriate phases. The last colon part where the microbial mechanism of digestion takes place includes only microbial enzymes and it was incubated overnight under the conditions of 37°C, pH 8, and 100 rpm.

The samples from each phase (1 mL) were mixed with cold ethanol (1:4; v/v) and incubated at -20°C for 1 h to precipitate proteins. After the incubation, samples were centrifugated for 30 min under the conditions of 4°C and 4000 rpm. The supernatant parts were removed and dried using a vacuum evaporator machine. The dry samples were dissolved with 600 µL $dH_2O$ and used in a phenol sulphuric acid assay to be quantified. As for the phenol-sulphuric acid assay, each 25 µL sample was mixed firstly with 25 µL phenol (1:1; v/v) and then 125 µL sulphuric acid in a plate. After the 20 min incubation at room conditions, concentrations were measured at $OD_{490}$ nm.

Table 4

Microorganisms And Their Enzymes For The Oral Phase

| **ORAL** | |
|---|---|
| **Mouth** | **Esophagus** |
| *Lactobacillus salivarus subsp. salivarius* ATP38112.1 | *Ligilactobacillus salivarus* ATP38122.1 |
| *Ligilactobacillus salivarius* ATP36889.1 | *Lactobacillus salivarus subsp. salivarius* ATP37586.1 |
| *Lactobacillus salivarus subsp. salivarius* ATP37244.1 | *Streptococcus intermedius* SQH51076.1 |
| *Streptococcus intermedius* SQH52440.1 | *Streptococcus intermedius* SQH51655.1 |

Table 5

Microorganisms And Their Enzymes For The Gastric Phase

| GASTRIC |
|---|
| *Lactobacillus rhamnosus GG* CAR86329.1 |
| *Bacteroides thetaiotaomicron* AAO77566.1 |
| *Bacteroides vulgatus* ABR41745.1 |

Table 6

Microorganisms And Their Enzymes For The Small Intestine Phase

| **SMALL INTESTINE** | | |
|---|---|---|
| **Duodenum** | **Jejunum** | **Ileum** |
| *Akkermansia muciniphila* ACD04858.1 | *Bifidobacterium longum subsp. infantis* ACJ51836.1 | *Bacteroides vulgatus* ABR38247.1 |
| *Bifidobacterium bifidum* BAQ98211.1 | *Bifidobacterium longum subsp. infantis* ACJ53413.1 | *Streptococcus thermophilus* SQF24907.1 |
| *Bifidobacterium bifidum* BAQ97897.1 | *Bacteroides uniformis* QQA29671.1 | *Streptococcus thermophilus* SQF25661.1 |
| *Lactobacillus brevis* ERK41518.1 | *Bifidobacterium catenulatum subsp. kashiwanohense* BAQ30021.1 | *Streptococcus thermophilus* SQF24918.1 |

Table 7

Microorganisms And Their Enzymes For The Large Intestine Phase

| LARGE INTESTINE | |
|---|---|
| **Proximal Colon** | **Distal Colon** |
| *Akkermansia muciniphila* ACD04701.1 | *Bacteroides vulgatus* ABR3893.1 |
| *Akkermansia muciniphila* ACD04208.1 | *Bacteroides thetaiotaomicron* AAO76145.1 |
| *Bifidobacterium bifidum* BAQ97280.1 | *Bifidobacterium longum subsp. infantis* ACJ53522.1 |
| *Bacteroides fragilis* CAH9389.1 | *Bifidobacterium lonfum subsp. infantis* ACJ51376.1 |

### 3.2.9. Statistical Analysis

Data obtained from this research was analysed statistically according to the one-way ANOVA variance analysis and Tukey's multiple comparisons statistical test to assess the statistical significance of the data at $p<0.05$ using NCSS 21 statistical software. Prior to ANOVA, the data were analyzed for normal distribution with the Shapiro–Wilk test, and for the homogeneity of variances using Levene's test.

### 3.2.10. Investigation of *N*-Linked Glycan Utilization by Selected Probiotics

*Lactobacillus rhamnosus GG, Levilactobacillus brevis, Lactobacillus salivarus subsp. Salivarius, Lactobacillus delbrueckii subsp. Bulgaricus, Bifidobacterium kashiwanohense, Bifidobacterium longum subsp. Infantis, Bifidobacterium bohemicum, Bifidobacterium pseudocatenulatum, Propionibacterium freudenreichii subsp. freudenreichii* were the bacteria strains used in this study. *Bifidobacterium* strains were grown under anaerobic conditions at 37 °C, by adding different concentrations (1%, 2%) of glycans as a carbon source to the RPMI 1640 no glucose medium with additional 0.05% cysteine, and medium containing tubes were incubated in an anaerobic jar (Anaerocult, Merk, Germany). *Lactobacillus* species were incubated in RPMI 1640 with three different glycan concentrations (0.5 %, 1%, 2%) at 37 °C under aerobic conditions. In addition, RPMI 1640 media without any carbon source used as negative control, and media containing 2% glucose was used as the positive control. *E.Coli XL1 Blue* used as the control pathogen that represents unbeneficial bacterium for analyzing consumption of pitaya *N*-glycans by pathogens.

Table 8

Selected Probiotic Strains, Collection Numbers And Biological Origins

| **Bacteria Strains** | **Collection** | **Isolation Source / Biological Origin** |
|---|---|---|
| *Lactobacillus rhamnosus GG* | ATCC 53103 | Infant, faeces |
| *Levilactobacillus brevis (Lactobacillus brevis)* | LMG6906 | Human, faeces |
| *Lactobacillus salivarus subsp. salivarius (Ligilactobacillus salivarius)* | LMG9477 | Saliva |
| *Lactobacillus delbrueckii subsp. bulgaricus* | LMG 6901 | Bulgarian yoghurt |
| *Bifidobacterium. kashiwanohense* | LMG 27585 | 1.5-year-old child, faeces |
| *Bifidobacterium longum subsp. infantis* | ATCC 15697 | Intestine of infant |
| *Bifidobacterium bohemicum* | LMG 27797 | Bumblebee (Bombus lucorum), digestive tract |
| *Bifidobacterium pseudocatenulatum* | LMG 10505 | Infant, faeces |
| *Propionibacterium freudenreichii subsp. freudenreichii* | LMG 16412 | Swiss cheese |

# CHAPTER 4
# RESEARCH FINDINGS

This chapter has presented qualitative and quantitative research findings addressing the research questions of this study.

## 4.1. Pitaya Protein Isolation Studies

Protein isolation from pitaya samples was performed by using alcohol precipitation as was described before. Separation of complex protein mixture in high resolution to acquire protein profile of pitaya and their molecular masses analysed by SDS-PAGE gel electrophoresis. In this study 3 µL from Protein Ladder (10-250 kDa) and different concentrations of protein samples as 5 µL, 10 µL and 15 µL purified protein samples loaded into the wells. After staining process diverse protein bands observed on gel. Intense protein bands were detected specifically around 55 kDa, and between 15-10 kDa. The protein profile of pitaya samples are shown in Figure 9.

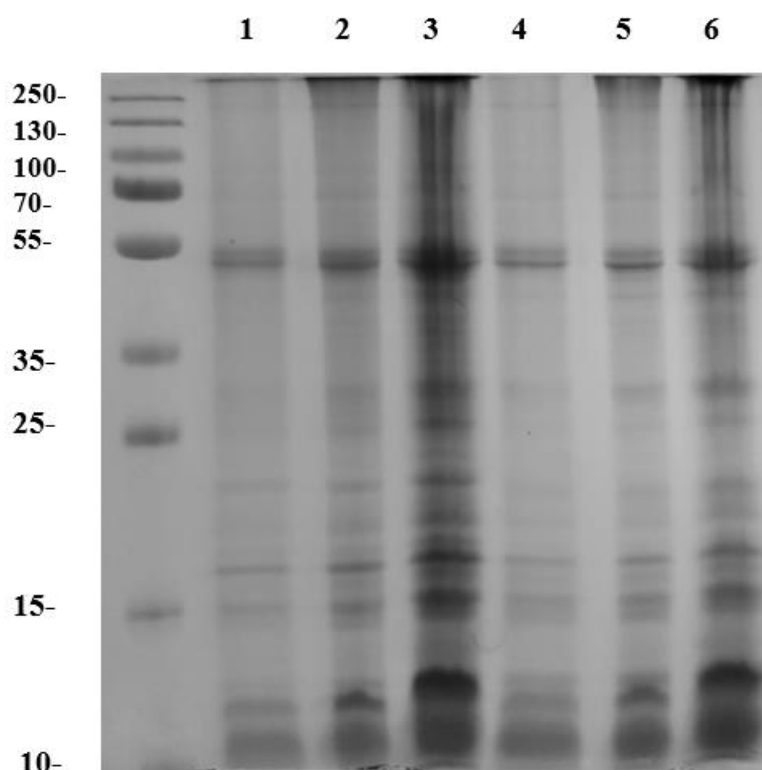



Figure 9. Visualization of total pitaya protein profile via SDS-PAGE gel (4-12%). After purification step, approx. 1–10 μg of protein was applied to gel, and visualized by commasie blue staining as described in the Method section. Lane 1,2,3: Imported Pitaya samples with 5 μL, 10 μL, 15 μL sample volume respectively; Lane 4,5,6: Local Pitaya samples with 5 μL, 10 μL, 15 μL sample volume respectively.

The abundance of proteins in samples can be estimated by the relative intensity of bands. High content of protein in sample indicated by thick bands and the thin bands point low amount of protein in sample. The protein distribution was observed homogeneously between local and important samples. However, it was observed that imported samples contain slightly more protein compared to local sample.

### 4.2. Detection of Protein Concentration by Qubit Assay

Total protein concentration of local and imported pitaya samples were detected by a fluorometric assay that can give results in a short time and at low concentrations. According

to results obtained, local pitaya has 7.8 mg/mL protein content while imported sample has 8.05 mg/mL protein concentration (Table 9).

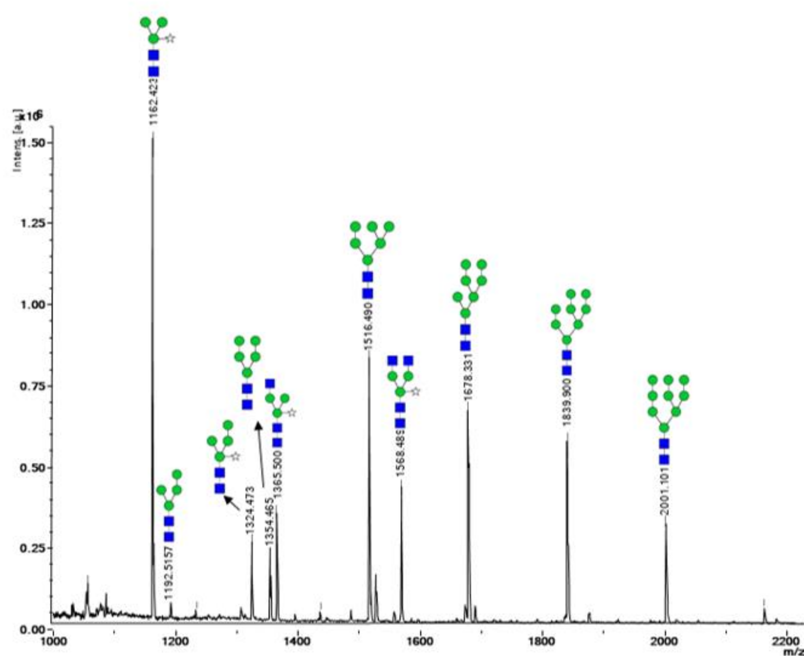
Table 9.

Protein Concentration of Pitaya Samples

| | Protein Concentration (mg/mL) |
|---|---|
| **Local Sample** | 7.8 |
| **Imported Sample** | 8.05 |

## 4.3. Confirmation of Pitaya N-Glycan Structures by MALDI-TOF-MS

**A**



**B**

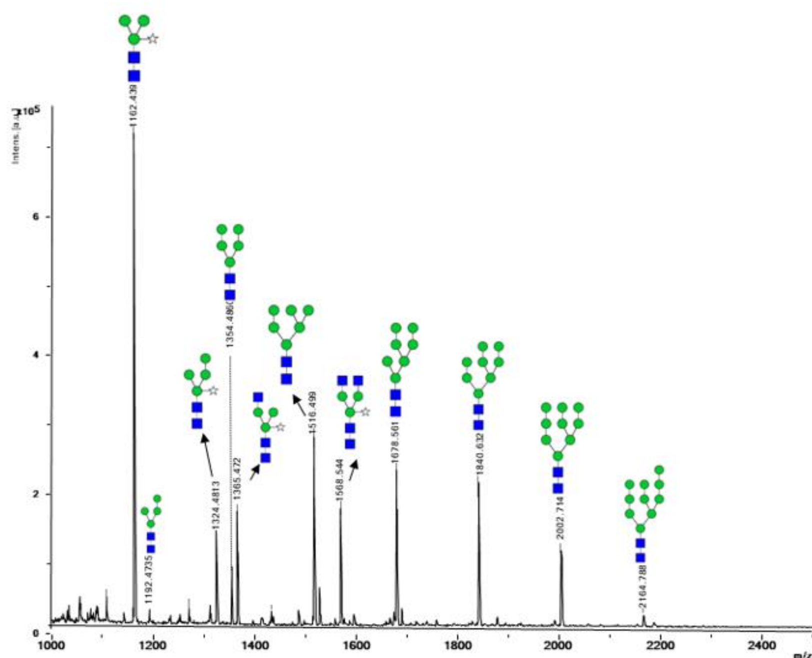



Figure 10. MALDI-TOF-MS Analysis Results of Local (A) and Imported (B) Pitaya N-Glycans

In order to confirm the glycan content and compare the glycan profiles of local and imported pitaya glycoproteins, firstly, the spectra were compared using the PNGase-F enzyme. Mass spectrometry results for pitaya shows neutral high mannosylated glycan structures and plant-originated pentose structures. This mass analysis are performed in MS-1 level.

### 4.4. Phenol Sulfuric Assay for Estimation of Glycan Release From Pitaya

Among the quantitative assays available for carbohydrate measurement, the phenol sulfuric acid method stands out as a superior choice due to its reliability and simplicity.. Phenol Sulfuric Acid microplate method was applied to confirm glycan release from Pitaya glycoproteins by using a glucose standard. In regard with the phenol-sulfuric acid assay, samples and standards was treated with of 5% phenol, concentrated $H_2SO_4$ in 1:1:5 ratio respectively. Following a 30-minute incubation at 35 rpm in the dark, samples were tested

for $A_{490}$, as the linearity range for total sugars of 10-100 ppm. All samples were prepared in duplicate.

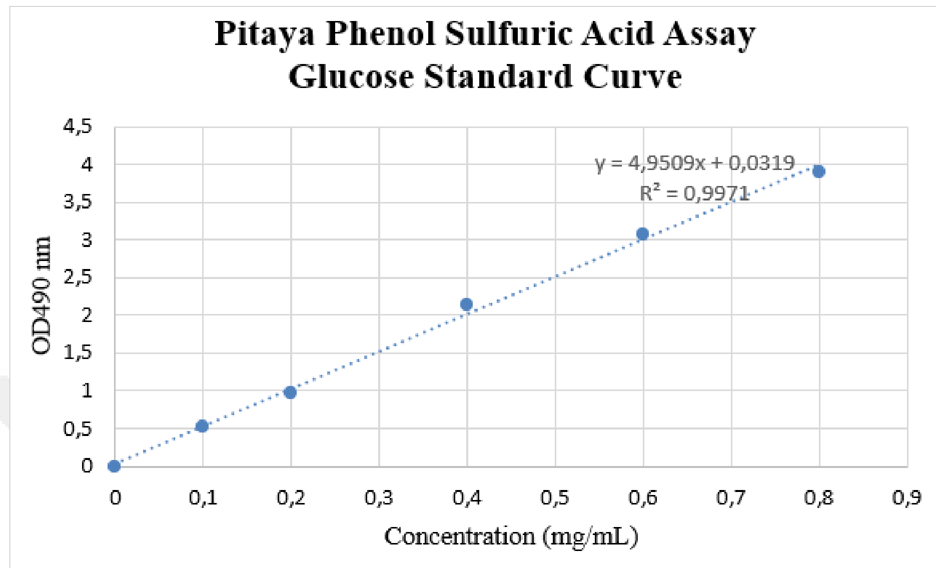



Figure 11. The phenol-sulfuric acid glucose standard graph created to determine the glycan concentration.

Table 10

Released Glycan Concentration From Dragon Fruit Glycoproteins

| | **Control (mg/mL)** | **EndoBI-1 Digested (mg/mL)** |
|---|---|---|
| **Local Sample** | 0.1345 | 0.2208 |
| **Imported Sample** | 0.1558 | 0.3277 |

### 4.5. *In-vitro* Digestion to Unleashing Power of Pitaya Glycoproteins

Pitaya glycoproteins were digested by an *in-vitro* digestion system with addition of microbial enzymes. The study aimed to ascertain the amount of glycoprotein digestion in the gastrointestinal system as well as the possible bioactive glycans generated during the process. According to the findings, glycan release was intensely determined in the small intestine and colon digestion phases for both imported and local samples. Results of the study

are provided below in Table 11. Samples separated from each digestion phase are shown in Figure 12.

Table 11

Glycan Concentrations After In-Vitro Digestion of Pitaya

| Released Glycan Concentrations from Pitaya Glycoproteins (mg/mL) | | |
|---|---|---|
| **Digestion Phases** | **Imported Mean ± SD** | **Local Mean ± SD** |
| **Control** | 0.1558 ±0.006 | 0.1345 ± 0.0007 |
| **Oral Phase** | 0.72 ± 0.29 | 0.18 ± 0.05 |
| **Gastric Phase** | 2.19 ± 0.31 | 0.94 ± 0.28 |
| **Intestinal Phase** | 4.22 ± 0.41 | 3.12 ± 0.19 |
| **Colon Phase** | 4.7 ± 0.79 | 3.57 ± 0.31 |

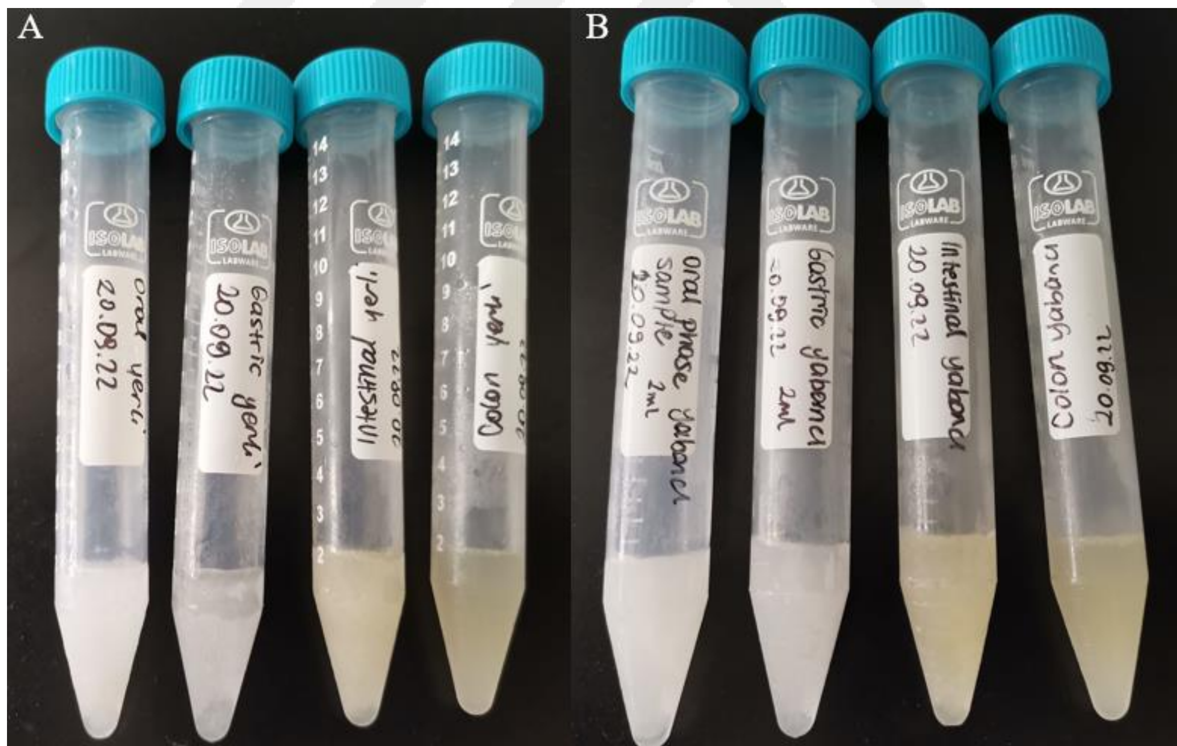



Figure 12. Pitaya Glycoproteins After Each Digestion Phase from Local (A) and Imported (B) samples.

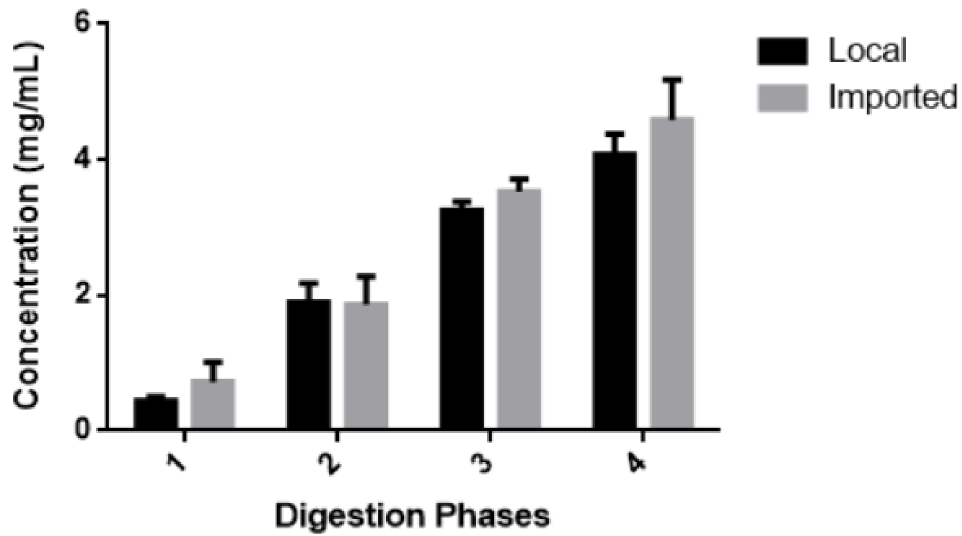



Figure 13. The concentration of released glycans after the in-vitro digestion process in each digestion phase. 1,2,3 and 4 indicate Oral, Gastric, Small Intestine, and Colon phases respectively. Data represent the mean of three experiments ± SD, and statistically significant difference between oral, gastric, small intestine, and colon groups was determined by ANOVA Tukey multiple comparison test.

### 4.6. Potential Prebiotic Activity of Pitaya *N*-glycans

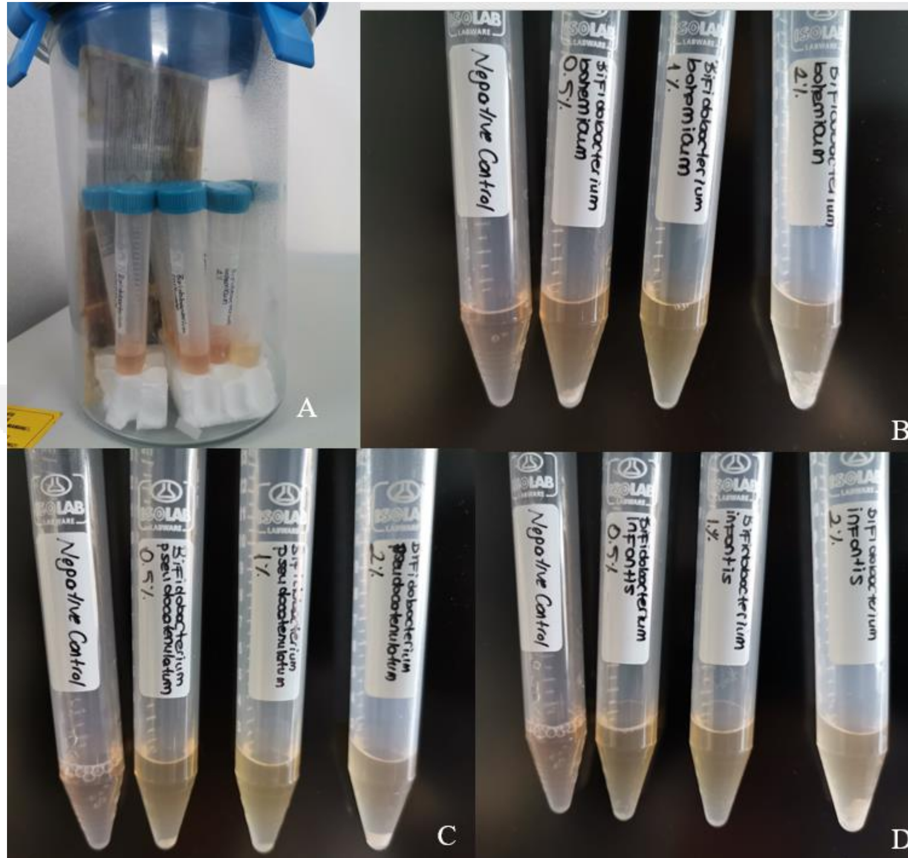



Figure 14. Bacterial Growth of Selected Anaerobic Bacteria. Figure shows the anaerobic jar conditions. (A) Anaerobic Jar; (B) Bifidobacterium Bohemicum; (C) Bifidobacterium pseudocatenulatum; (D) Bifidobacterium infantis

Glycans have been described as a proper source of prebiotics with several studies as explained in introduction section. In this research, we aimed to enlighten prebiotic potential of dragon fruit glycans.  In the presence of three different glycan concentrations, selected three *Lactobacillus spp*. strains grew similarly. *Propionibacterium freudenreichii subsp. Freudenreichii* shows a concentration-dependent increase without any dramatic difference. The lowest bacterial growth among probiotic strains observed in Bifidobacterium spp (Figure 14). Abundance of bacterial strains in presence of candidate *N*-glycans range between 0.051 and 0.911 ($OD_{600}$). However, the growth of *Lactobacillus Rhamnosus GG,*

*Lactobacillus brevis* and *Lactobacillus salivarus spp.* significantly higher. Glucose was used as a positive control to compare carbon sources, all bacterial species showed successful growth in the presence of glucose. Media that did not contain any carbon source other than Pitaya glycans were used as negative control. *E.coli XL1 Blue* showed similar growth with Bifidobacterium species, but significantly less growth than *Lactobacillus spp.*

Table 12

Bacterial growth ($OD_{600}$) according to three different glycan concentrations.

| | **Glycan Concentration** | | | | |
|---|---|---|---|---|---|
| | **Positive Control** | **2 %** | **1%** | **0.5%** | **Negative Control** |
| ***Bacteria Strain*** | **Growth** | | | | |
| *Lactobacillus rhamnosus GG* | 1.701 | 0.875 | 0.831 | 0.911 | 0.043 |
| *Lactobacillus brevis* | 1.492 | 0.899 | 0.882 | 0.879 | 0.061 |
| *Lactobacillus salivarus subsp. salivarius* | 1.057 | 0.792 | 0.75 | 0.641 | 0.044 |
| *Propionibacterium freudenreichii subsp. freudenreichii* | 0.641 | 0.437 | 0.399 | 0.367 | 0.089 |
| *Bifidobacterium longum subsp. infantis* | 0.621 | 0.128 | 0.08 | 0.051 | 0.057 |
| *Bifidobacterium bohemicum* | 0.677 | 0.179 | 0.041 | 0.1 | 0.039 |
| *Bifidobacterium pseudocatenulatum* | 0.652 | 0.228 | 0.198 | 0.185 | 0.033 |
| *E. coli XL1 Blue* | 0.587 | 0. 322 | 0.304 | 0. 273 | 0.041 |

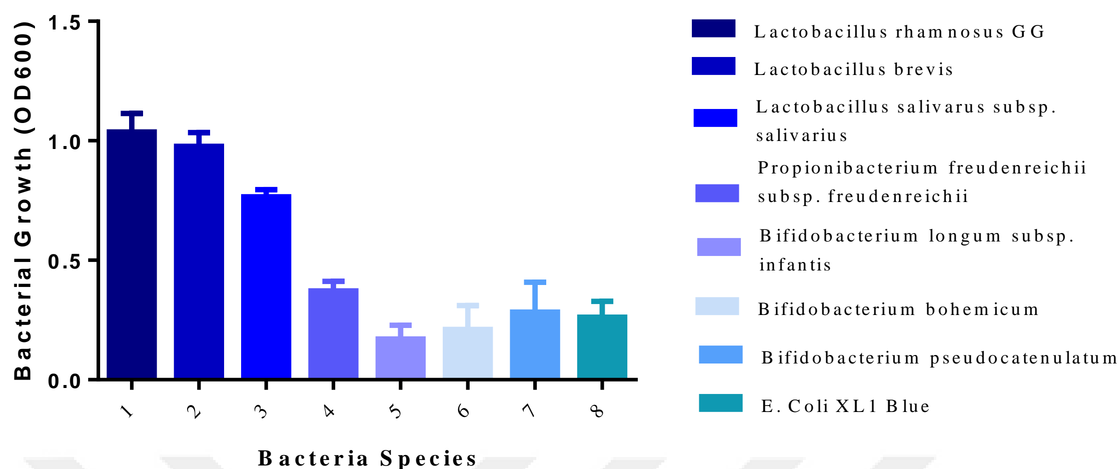



Figure 15. Average growth of bacterial species. Numbers indicated bacterial species shown in the right side of the graph respectively.

# CHAPTER 5
# RESULTS AND RECOMMENDATIONS

Fruits have been known as beneficial and nutritious food sources since ancient times. Today, the consumption of pitaya fruit, is at the top of the fruit consumption, which has increased rapidly as a result of the orientation to natural resources. This remarkable increase in consumption can be attributed to the growing inclination towards natural resources and the desire for health-promoting food choices. Pitaya fruit has gained significant popularity due to its perceived nutritional value and positive associations with overall well-being. Pitaya can be considered a new fruit for several counties lately since production and consumption are progressively increasing as an alternative crop. A compelling need exists to thoroughly investigate the biomolecular composition of regularly consumed foods to unveil their intricate interaction with the human body and additional foreign xenobiotics. Various studies with pitaya fruit draw attention to the positive effects of pitaya on many ailments, including cancer and gastrointestinal disorders. However, the bioactive components and effects of this fruit have not been fully elucidated today. There is a profound lack in the literature on the content of glycans in pitaya fruit, which is known to have many benefits on intestinal health, and its effects on the digestive system. This imperative necessity of meticulous research includes comprehensive analysis and scrutiny.

The first part of the thesis covers the protein isolation and determination of protein concentration obtained from pitaya fruit samples. To uncover the total protein profile of pitaya, samples were homogenized in chemical and physical ways. Subsequently, isolated proteins were visualized and profiled by SDS-PAGE on different concentrations. As a result of this investigation, we discovered that pitaya fruit contains a wide range of protein structures. Retrospective studies have searched the pitaya proteins to a very limited extent. No study was found in the literature that showed the total protein content of pitaya by SDS-PAGE imaging. However, when compared with quantitative data, studies have stated that 100 g of pitaya contains between 0.4 g and 2.2 g of protein (Attar et al., 2022). When this range is taken as a reference, our results show an accuracy of 0.78 g and 0.8 g for local and imported samples, respectively. Based on the findings obtained from analysis, it has been concluded that imported sample exhibits a slightly higher protein content than the locally

sourced sample. However, it is noteworthy that the disparity between the two samples is not statistically significant. Also, no remarkable variation was observed when the bands were compared between the two samples.

While a comprehensive understanding of all protein contents found in the pitaya remains incomplete, certain investigations have highlighted the isolation of enzymes such as amylase with a molecular weight of 42.1 kDa, and pectinase with a weight of 34.2 kDa from pitaya (Amid and Manap, 2014; Amid et al., 2014). The gel images acquired in this study substantiate the presence of prominent bands, particularly at the amylase level. Nevertheless, it is imperative to conduct further analysis in order to discern the precise identification of individual proteins.

Since 80 % of eukaryotic proteins have been detected previously as in N-glycosylated form, it becomes imperative to comprehend the functionality and potential impact of these attached N-glycans when consumed by the human body. EndoBI-1 as a human microbiome derived glycosyl hydrolase enzyme was used the release N-glycans derived from dragon fruit proteins. The concentration of the released glycans was quantified using the phenol sulfuric acid method, which is widely regarded as the most precise and user-friendly technique for carbohydrate detection, ensuring accurate and reliable results. According to the results, when the glycan concentrations of pitaya samples with similar protein concentrations are compared, it is notable that significantly higher amounts of glycans were observed in local samples than in imported samples. Furthermore, the identification of certain carbohydrates in the control portion of the samples, which underwent purification using 10 kDa filter tubes to remove free glycans subsequent to the glycan release process, suggests the potential presence of sugar molecules that fall below the 10 kDa threshold.

The comprehension of the fate of food components can be achieved by simulating these digestion models in-vitro. As an uncovered research topic, in this part of the thesis, the aim was to determine the fate of proteins after being digested with a new digestion model that includes novel microbial enzymes. This model enables us to reach a novel glycan data after glycoprotein consumption by microorganisms. Additionally, it is the first time this

novel model used for plant-derived proteins. For this research, local and imported, both pitaya glycoproteins were used for the purpose of digestion.

Mass spectrometry analysis used to verify the glycan content and conduct a comparative analysis of glycan profiles between locally sourced and imported pitaya proteins, the initial step involved a thorough comparison of spectra via PNGase-F enzyme. Subsequent to this enzymatic treatment, the resulting mass spectrometry outcomes for pitaya revealed the presence of neutral high mannosylated glycan structures, alongside pentose structures originating from plants. It is important to note that the aforementioned mass analysis was conducted at the MS-1 level. Based on the conducted analysis, it has been ascertained that the N-glycan contents derived from the pitaya samples exhibited similar profiles.

In order to evaluate the significant difference in glycan release within the digestive phases of local and imported samples, we first determined their glycan concentrations. According to measured optical density ($OD_{490}$) values after phenol sulfuric acid assay glycan concentration was calculated for each digestion phase. Obtained results from in-vitro digestion of local protein samples indicated a statistically significant difference between groups of the oral, gastric, small intestine, and colon was determined by ANOVA, Tukey multiple comparison test ($p < 0.05$). There is significantly different glycan release within ( $p < 0.001$) for all compared to digestion phases of the local protein sample and except for the oral phase ($p > 0.05$). All other phases were also found significantly different between control ($p < 0.001$). Glycan release between oral, gastric, and intestinal phases of the imported protein samples was found significantly different ($p < 0.001$). The statistical results obtained show that the least release is in the oral and gastric phase as expected, and the most release is in the small intestine and colon regions where bacteria-secreting glycosyl hydrolase enzymes are concentrated. In order to compare the glycan release resulting from in-vitro digestion of protein samples obtained from local and imported pitaya, an equal variance t-test was used to reveal the differences between the two samples. As a result of the analysis, oral, gastric, intestinal, and colon phases compared for the two samples were not found significantly different from each other. Accordingly, results indicate there is no different glycan levels were found in the local sample and imported sample.

Regarding the results of the prebiotic activity tests, selected strains' growth performance was examined in relation to the effect of candidate prebiotic glycans. Prebiotics are considered to have a favorable effect on the host's health and to be consumed preferentially by beneficial bacteria. Additionally, the prebiotic component needs to improve the probiotic bacteria' development and activity. The experiment was designed for three different glycan concentrations (2%, 1%, and 0.5 %) of pitaya fruit proteins with the combination of local and imported proteins. Based on the findings, growth of *Lactobacillus spp*. and *Propionibacterium freudenreichii subsp.* were found significantly increased. In contrary, Bifidobacterium species have no significant growth with candidate glycans and when compared to *E.coli* they grew similarly. These results suggest glycans may have a selective prebiotic effect on Lactobacillus species. With these findings, we reach the prebiotic effect of pitaya N-glycans on top of the free oligosaccharides that were previously analysed in the literature (Khalili et al., 2014). However, an accurate representation of the complicated microbiological processes taking place within the human body can be achieved by taking into account the complex interactions and dynamics within a co-culture. Therefore, developing a co-culture system under the proper circumstances is essential to investigate this analysis further. Additionally, it is necessary to expand this study by increasing the number and species of probiotic and pathogen bacteria.

The microbial enzyme-based in-vitro system performed in this study was tested on plant-derived proteins for the first time. Thus, the activities of these unique enzymes used have been proven on plant-based proteins. However, determining the action mechanisms and activity tests of these novel enzymes in future studies will shed light on both this research and candidate studies. Since human body inhabited a vast number of microorganism that affects digestion directly or indirectly this results cannot fully mimic the whole bacterial enzymes that released by the microorganisms in the body. However, findings of this study are anticipated to spur additional investigation and advance our knowledge of the intricate procedures entailed in glycan metabolism and digestion. Understanding the complexity of glycobiology and its consequences for numerous facets of human health and nutrition can be improved by deepening our understanding in this field. The findings of this study will stimulate advancement and innovation in the field of in vitro digestion of dragon fruit and its interaction with glycobiology, which will motivate and direct future research.

Researchers can gain insight into the complex pathways involved in glycan usage by studying how microbial enzymes break down and use complex carbohydrates. This information could impact many scientific fields, including environmental microbiology, agriculture, and biotechnology. In this regard, a thorough comprehension of the effective degradation of plant glycoproteins by particular bacteria may open the door like novel biofuel production techniques.

Investigating the prebiotic effects of fruit glycans has important ramifications for microbial research and human health. Prebiotics significantly impact many aspects of human well-being because they promote the growth and activity of good gut flora. Pitaya glycans have the potential to influence the makeup and functionality of the gut microbiota as prebiotics as it is shown in this study.

For the food industry, investigating the prebiotic potential of fruit glycans is promising. Finding particular fruits or fruit extracts with advantageous prebiotic qualities could guide the creation of functional foods or dietary supplements meant to encourage the best possible gut health. In the field of medicine, understanding how glycans and microbial enzymes interact can be crucial for the creation of specialized therapeutic approaches. Additionally, the knowledge gained from researching prebiotic effects and glycan usage has significant ramifications for specific therapy. A customized strategy to diet and medicinal therapies that take into account particular glycan consumption and prebiotic requirements might be designed given the personalized nature of gut microbiota regulation and activity. In this context, it is thought that the glycans to be produced within the scope of the thesis have potential contribute to various treatments in future biotechnological development studies of natural origin. In many cases, the consumption of different drugs with fruits has known effects on both the bioavailability level of the drugs and the level of absorption in the intestine, causing the inhibition of various enzymes and giving rise to serious toxic effects. As in the grapefruit example, it is thought that the impact of glycoprotein-based mechanisms may be revealed and clinical effects can be observed in future studies. The investigation into the complex nature of fruit-drug interactions and their potential influence on the therapeutic consequences of pharmaceuticals can be enhanced partially by the findings on the pitaya fruit.

In conclusion, using scientific data to research how microbial enzymes use glycans and investigate the prebiotic effects of fruit glycans is an essential step in expanding the field of glycobiology. The gained knowledge has the potential to alter several fields, including the food, pharmacy, and the field of customized medicine, leading to improvements in people's health and well-being. On the basis of results obtained from this thesis, enligted to certain molecular and biological properties of pitaya fruit.

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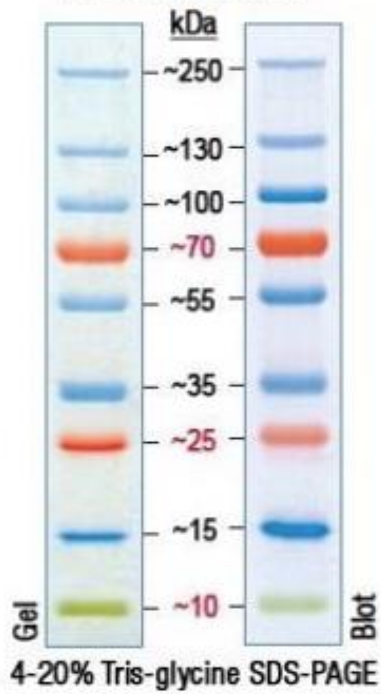
## APPENDICES

## APPENDIX 1

## PROTEIN LADDER USED FOR SDS-PAGE PROTEIN ANALYSIS



# APPENDIX 2

## BUFFERS USED FOR *IN-VITRO* DIGESTION METHOD

| **Chemical** | **mol/L (M)** | **SSF (pH:7)** | **SGF (pH:3)** | **SIF (pH:7)** |
|---|---|---|---|---|
| **KCl** | 0.5 | 15.1 mL | 6.9 mL | 6.8 mL |
| **$KH_2PO_4$** | 0.5 | 3.7 mL | 0.9 mL | 0.8 mL |
| **$NaHCO_3$** | 1 | 6.8 mL | 12.5 mL | 42.5 mL |
| **NaCl** | 2 | - | 11.8 mL | 9.6 mL |
| **$MgCl_2(H_2O)_6$** | 0.15 | 0.5 mL | 0.4 mL | 1.1 mL |
| **$(NH_4)_2CO_3$** | 0.5 | 0.06 mL | 0.5 mL | - |
| **HCl** | 6 | 0.09 mL | 1.3 mL | 0.7 mL |
| | 0.3 | | | |

# APPENDIX 3

**BIOGRAPHY**