T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

PROTEIN ISOLATION AND *N*- GLYCAN CHARACTERIZATION OF DIFFERENT MUSHROOM SPECIES BY USING PNGASE F ENZYME

MASTER OF SCIENCE THESIS

TUBA ÇAĞIRTEKİN

Thesis Supervisor

ASSOC. PROF. SERCAN KARAV

ÇANAKKALE – 2023

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This work is supported by Canakkale Onsekiz Mart University
The Scientific Research Coordination Unit
Project number: FYL-2022-3873

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CANAKKALE ONSEKİZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

The study titled "Protein Isolation and *N*- glycan Characterization of Different Mushroom Species by Using PNGase F Enzyme", prepared by Tuba ÇAĞIRTEKİN under the direction of Assoc. Prof. Sercan KARAV and presented to the following jury members on 31/08/2023 was unanimously accepted as a MASTER THESIS at Canakkale Onsekiz Mart University, School of Graduate Studies, Department of Molecular Biology and Genetics.

……	**Jury Members**	**Signature**	..…...
	Assoc. Prof. Sercan KARAV (Supervisor)	……	
	Prof. Dr. Kemal Melih TAŞKIN (Member)	……	
	Assoc. Prof. Hacı Mehmet KAYILI (Member)	……	

Thesis No : 10571026

Thesis Defence Date : 31.08.2023

……

Prof. Dr. Ahmet Evren ERGİNAL

Director

18/09/2023

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Tuba ÇAĞIRTEKİN

31.08.2023

ACKNOWLEDGEMENT

I would like to thank my respected advisor, Assoc. Prof. Sercan KARAV, I would like to express my endless thanks to my teammates who have faced all the difficulties with me during the study, to my dear sister Merve ÇAĞIRTEKİN and my brother Vedat ÇAĞIRTEKİN who supported me in every stage of my life. I am thankful for the financial support of COMU The Scientific Research Coordination Unit (Project number: FYL-2022-3873).

Tuba ÇAĞIRTEKİN

Çanakkale, August 2023

PROTEIN ISOLATION AND *N*- GLYCAN CHARACTERIZATION OF DIFFERENT MUSHROOM SPECIES BY USING PNGASE F ENZYME

ÖZET

Tuba ÇAĞIRTEKİN
Çanakkale Onsekiz Mart Üniversitesi
Lisansüstü Eğitim Enstitüsü
Moleküler Biyoloji ve Genetik Anabilim Dalı Yüksek Lisans Yeterlik Tezi
Danışman: Doç. Dr. Sercan KARAV
31/08/2023, 42

Yenilebilir mantarlar çok eski zamanlardan beri tüketilmekte ve özel bir yiyecek türü olarak sınıflandırılmaktadır. Özel aromaları dışında yüksek besin kalitesine sahip ve birçok farklı biyoaktif bileşik içerirler. Onlara antikanser, antibiyotik ve antioksidan özellikler gibi sağlığa faydalı birçok özellik kazandıran yüksek miktarda protein ve esansiyel amino asitler içerdikleri bulunmuştur. Her mantar türünün protein içeriği türüne bağlıdır ve sağlığa yararlı özelliklerini etkiler. Bu nedenle farklı mantar türlerinin her bir protein parçasının ayrı ayrı araştırılması önemlidir.

Protein glikozilasyonu; protein aktivitesini, stabilitesini ve protein-protein etkileşimlerini etkileyen translasyon sonrası bir modifikasyondur. Glikanlar; proteinlerin stabilizasyon, konformasyon ve katlanma özelliklerini etkiler. Glikanların etkilerini incelemek ve işlevlerini anlamak için bağlandıkları proteinlerden ayrılmaları gerekir. Deglikosilasyon, glikanları bağlı oldukları glikoproteinlerden ayırma işlemidir. Peptid-*N*-glikosidaz F (PNGase F), *N*- glikanların glikoproteinlerden deglikosilasyonunu katalize etmek için kullanılan bir enzimdir. Son zamanlarda serbest *N*- glikanların prebiyotik kaynak olarak kullanılabileceği bildirilmiştir.

Bu çalışma, PNGase F enzimini kullanarak üç farklı yenilebilir mantar türünün (*Marasmius castaneophilus*, *Agaricus bisporus*, *Pleurotus ostreatus*) glikoproteinlerini deglikolize etmeyi amaçlamaktadır. Enzim, mantar glikoproteinlerini parçalar ve serbest *N*-glikanlar elde edilir. Bu *N*-glikanların karakterizasyonu, MALDI-TOF Kütle spektrometrisi kullanılarak yapılmıştır. Elde edilen *N*-glikanlar, potansiyel birer prebiyotik adaylarıdır ve

prebiyotik aktivitelerinin test edilmesi için Lactobacilli ve Bifidobacterium gibi farklı probiyotik bakterilerin bulunduğu bir ortamda bakterilerin bu glikanlardan faydalanması durumuna göre incelenmiştir.

Anahtar Kelimeler: Yenilebilir mantar, *N*-glikan, MALDI TOF, prebiyotik, PNGase F.wo

PROTEIN ISOLATION AND N- GLYCAN CHARACTERIZATION OF DIFFERENT MUSHROOM SPECIES BY USING PNGASE F ENZYME

ABSTRACT

Tuba ÇAĞIRTEKİN
Çanakkale Onsekiz Mart University
School of Graduate Studies
Master of Science Thesis in Molecular Biology and Genetics
Supervisor: Assoc. Prof. Sercan KARAV
31/08/2023, 42

Edible mushrooms have been consumed since ancient times and are classified as a special kind of food. Except for their special aroma, they have high nutritional quality and contain many different bioactive compounds. It has been found that they contain high amount of proteins and essential amino acids which give them many health-beneficial features such as anticancer, antibiotic, and antioxidant properties. The protein content of each mushroom species depends on the species and affects its health- beneficial properties. Therefore, it is important to investigate each protein fragment of different mushroom species separately.

Protein glycosylation is a post-translational modification that affects protein activity, stability, and protein-protein interactions. Glycans affect the stabilization, conformation, and folding properties of proteins. In order to examine the effects of glycans and to understand their functions, they must be separated from the proteins to which they are attached. Deglycosylation is a process of separating glycans from glycoproteins. Peptide- *N*- glikosidase F (PNGase F) is a mainly used enzyme that catalyzes the deglycosylation of *N*- glycans from glycoproteins. Recently, it has been reported that free *N*- glycans can be used as prebiotic sources.

This study is aimed to deglycolyze glycoproteins of three different edible mushroom species (*Marasmius castaneophilus, Agaricus bisporus, Pleurotus ostreatus*) by using PNGase F enzyme. The enzyme cleaves the fungal glycoproteins and free *N*-glycans are obtained. *N*- glycans was charactarized by using MALDI-TOF Mass

spectrometry. Obtained *N*- glycans are potential prebiotic candidates and their prebiotic activity tested in an environment with different probiotic bacteria such as Lactobacilli and Bifidobacteria, depending on whether the bacteria benefit from these glycans.

Keywords: Edible mushroom, *N*- glycan, MALDI TOF, prebiotic, PNGase F.

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SYMBOLS and ABBREVIATIONS

%	Percent
Asn	Asparagine
A. bisporus	*Agaricus bisporus*
B. bifidum	*Bifidobacterium bifidum*
B. bohemicum	*Bifidobacterium bohemicum*
B. infantis	*Bifidobacterium infantis*
B. kashiwanohonse	*Bifidobacterium kashiwanohonse*
dH_2O	Distilled water
et.al	Others
g	Gram
GI	Gastrointestinal
L. brevis	*Lactobacillus brevis*
L. bulgaricus	*Lactobacillus bulgaricus*
L. rhamnosus	*Lactobacillus rhamnosus*
MALDI-TOF MS	Matrix Assisted Laser Desorption- Ionization- Time of Flight Mass Spectrometry
M. castaneophilus	*Marasmius castaneophilus*
mg	milligram
mg/mL	Milligram per milliliter
mL	Milliliter
OD	Optical Density
PNGase F	Peptidyl N-Glycosidase F
P. ostreatus	*Pleurotus ostreatus*
SDS- PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Thr	Threonine

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CHAPTER 1
INTRODUCTION

Edible mushrooms have been consumed since ancient times due to their delicious taste and high nutritional content. Their nutritional ingredients include bioactive compounds such as proteins, polysaccharides, β- glucans and lipids that give them health-promoting properties. Health beneficial features of mushrooms can be listed as antioxidant, antimicrobial, anticancer, antibiotic and prebiotic effects. These properties are affected by fungal species in which the protein and glycan content varies. Therefore, it is important to isolate their proteins and investigate each protein fraction in detail.

Edible mushrooms contain high amounts of protein, essential amino acid and low amounts of fat, making them an important source of vegan protein. The fact that mushrooms can be produced easily and cheaply, the pH and thermal stability of their proteins are high, and that they can be grown even in areas with industrial wastes have attracted the attention of both the food industry and the scientific community. High protein content (19-40% on a dry basis), aroma, and fibrous texture of edible mushrooms; promoted their use as a substitute for animal protein. They are important sources of protein compared to most vegetables and contain eight essential amino acids that make up 25–45% of total amino acids.

Many proteins in nature are found as glycoprotein form. Post-translation modification of proteins through glycosylation involves attachment of small carbohydrates, known as glycans, which subsequently lead to changes in the conformation, function, stability and interaction with other proteins. Most glycans are attached to proteins on asparagine residues in eukaryotes, and this process is called *N*-linked glycosylation. Separating glycans from the proteins to which they are bound is known as Deglycosylation, allowing for an in-depth examination of the glycans. Nowadays, it has been found that glycans can be great prebiotic canditates.

The branching, structure, solubility, molecular weight, ligands such as protein or peptide of glycans isolated from many different fungal samples were found to be variable. Fungal glycans are generally separated from sugar compounds such as glucose, fructose, galactose, mannose, arabinose, and glucuronic acid. The well-known fungal glycans are β-glucans and mannoses. Different purification methods result in the production of varying glycan fractions. Recently, enzyme extraction is a widely-used method because it allows

the separation of different glycan structures and can be applied more easily than other methods. Especially, PNGase F enzyme isolated from the bacteria *Flavobacterium meningosepticum* is widely used because it catalyzes the separation of different types of *N*-glycans.

1.1. Importance of Proteins and Aminoacids

All living organisms are compost of proteins. They account for over 50% of the dry weight of cells. They provide many essential funtions for human such as digestive enzymes play roles on facilitating chemical reactions, hormones help to coordinate the body, antibodies help to support immune functions. They have roles on cell to cell interactions, cell to microbe interactions and microbe- microbe interactions.

Proteins are needed to be hydrolized by proteases and peptidases to create a nutritional value. Dietary proteins are hydrolized by digestive enzymes to produce aminoacids, tripeptides and dipeptides in the gastrointestinal lumen (Wu, 2016). They provide essential aminoacids and organic nitrogen. Essential aminoacids are used for protein synthesis which proteins maintaince growing and repairing of tissues. They also provide nitrogens which are essential for synthesis of nitrogen-containing compounds such as nucleic acids, purines, pyrimidines and various neurotransmitter molecules.

Proteins are made up of aminoacid building blocks. There are 20 aminoacids which are bounded with peptide bonds. There are three types of aminoacids; Essential aminoacids, non essential aminoacids and semi essential aminoacids. Nine essential amino acids, including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, are crucial for the body's function. These essential amino acids cannot be synthesized in the body and therefore have to be taken into the body from outside. Amino acids are fundamental precursors for the body because they provide nitrogen and sulfur which molecules can not be synthesized in the body and can not be taken from other nutritional substances such as carbohydrates and fats. Amino acids are used for synthesis of glutathione, dopamine, nitric oxide, DNA and RNA which molecules have huge impact on physiological mechanisms. Glutamine, aspartate and glutamate are synthesized by amino acids and are utilized in small intestine to provide eneregy demand of the body. Therefore, aminoacids have huge effects on the body and deficiency of them causes some health diseases such as kwashiorkor and marasmus.

In recent years, the requirement for essential amino acids and proteins by humans is being a major interesting topic. Protein-rich diets increase satiety and provide a thermogenic effect when compared with carbohydrate and fat-rich diets.

1.1.1. Importance of Glycoproteins and Deglycosylation

Glycans are small carbohydrate molecules thhat generally found on the outer surface of cellular and secreted macromolecules (Reily et al., 2019). Glycans can be found in various cell types such as animals, plants, fungi and viruses. Glycans have important roles in cellular responses to environmental stimuli, cell growth and cell differentiation (Varki, 2017). Glycans can covalently bind to lipids or proteins by the process of glycosylation. Glycosylation is one of the most common post-translational modification processes which is playing the role in binding small carbohydrate units, glycans, to proteins, lipids or other saccharides (Schmelter et al., 2023). Glycosylation occurs in endoplasmic reticulum and golgi part of the cells. Glycosyltransferase and glycosidase enzymes catalyze this mechanism to create glycoconjucates. The constituent glycan components and their combinations, attachment positions, and branching types affect the functions and roles of these "glycoconjugates".

When small carbohydrate molecules, glycans covalently bind to to a hydroxyl group of a protein, it is called glycoprotein. Most of the proteins in nature are found as glycosylated. Almost half of the proteins in the body require glycosylation to carry out their biological activities. Protein glycosylation affects the activity, stability, and interactions of the protein. Most of the proteins on the outside of eukaryotic cells contain covalently linked glycans. Their structural roles; mediating interactions with the environment, facilitating correct folding as well as direct interaction with lipids.

Synthesis of glycoproteins are occured at Endoplasmic reticulum and golgi aparatus (Davis, 2002). Glycans bind to proteins via co-translation and post-translation. The ribosome which contains mRNA that codes for proteins attaches to the endoplasmic reticulum. Once the proteins are synthesized, they enter the lumen of the endoplasmic reticulum. The glycosylation of these proteins occurs by the addition of glycan moieties to the proteins with the help of glycosyl transferases. First, glycans are synthesized by glycosyltransferases and then attached to proteins. Then, glycoproteins enter the Golgi apparatus which trims the carbohydrate residues through the activity of glycosidases. Trimmed carbohydrate residues bind to the glycoproteins to obtain mature glycoproteins.

The constituent monosaccharide components and their combinations, attachment positions, and branching types influence the functions and roles of glycoproteins. Since the number of sugar moieties that attach to proteins is various, glycosylation process provides diversity to the proteome (Eichler, 2019). These sugar moieties also have many isomeric structures, so they create variation among the protein structures to which they are attached. There are two types of glycans in eukaryotic systems, *N*- and *O*- glycans. *N*-glycans bind to asparagine residues of proteins while *O*- glycans bind to serine or threonine residues of proteins. Most of glycoproteins in nature are found in the *N*- linked glycosylated form. *N*-glycans influence numerous features of their bound glycoproteins, such as conformation, solubility, antigenicity, activity, and recognition by other glycan-binding proteins. *N*-glycans must be attached to at least one amino acid sequence formed as Asn-X-Ser/Thr, where "X" is any amino acid other than Pro. Whereas, all asparagine amino acids are not glycosylated in this aminoacid sequence. This sequence(Asn-X-Ser/Thr) can be used as a marker to detect the location and orientation of the attached glycoprotein throughout the cell.

All eukaryotic *N*- glycans have a common sequence Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1–4GlcNAcβ1–Asn-X-Ser/Thr. *N*-glycans are classified into three types;

- Oligomannose: only Man residues extend the sequence.
- Complex: the "antennae" initiated by GlcNAc extend the sequence.
- Hybrid: Man sequence extends the Manα1-6 arm and one or two GlcNAc elongates the Manα1-3 arm.

Deglycosylation process is used for examining properties of proteins without bound glycans. In this way, proteins that do not contain glycans and have new properties can be obtained. There are various chemical and enzymatic separation methods have been used for releases glycans from glycoproteins (Triguero et al., 2010).

1. Chemical deglycosylation: It is cheap, easy and substrate specific method. It does not cause any physicochemical deterioration in the polypeptide chain. β-elimination and hydrazination are the most used chemical deglycosylation techniques. The β-elimination method uses alkaline conditions to obtain glycans from glycoproteins. However, the use of alkaline conditions can damage the structure of the resulting glycans. Hydrazination method uses hydrazine for the hydrolysis reaction of glycoproteins. Both methods have

disadvantages such as the use of hazardous chemicals and damage to the structure of glycoproteins and glycans.

2. Enzymatic Deglycosylation: Commercial deglycosydase enzymes are used for separation of glycans from glycoproteins. Peptidyl-*N*-glycosidases (PNGase) are enzymes for releasing *N*- glycans from bound glycoproteins. These enzymes hydrolyze the amide side of the polypeptide chain to obtain asparagine-linked glycans. Numerous different sized and charged *N*- glycans can be obtained by this method. The only drawback is the heat denaturation required prior to the enzymatic reaction to ensure effective glycan release.

To understand the functions and structures of *N*- glycans, they must be released from their bound proteins. Deglycosylation is the separation of glycans from glycoconjugates. It is used for analysis of pure glycans. The enzymatic deglycosylation method is the most useful method to obtain pure and different types of *N*-glycans. They can be separated from glycoproteins via various deglycosylase enzymes. Peptide- *N*-glycosidase F (PNGase F) enzyme is a deglycosylase enzyme that used for releasing of all types of *N*- glycans such as hybrid, complex and oligomannose. This enzyme is isolated from *Flavobacterium meningosepticum* which is a gram-negative bacterium (Norris et al., 1994). This enzyme cleaves the C-N bond of the glycosylated asparagine side chain, changing asparagine to aspartic acid and freeing ammonia and intact glycan. Due to the association of this activity with lysosomal glycosylaseparaginases in mammalian and bacterial cells, it is called amidase or amidohydrolase.

Mass spectrometry is one of the outstanding methods to indicate protein glycosylation by providing analysis of oligosaccharides or glycopeptides resulting from enzymatic digestion (Morelle et al., 2009). This method provides two basic sets of information. First, it provides the glycan analysis of a glycoprotein, giving a detailed view of the topology, branching, and connectivity of structures found in that protein. Second, it provides the analysis of glycopeptides, providing information on the extent and site-specific glycan composition of a glycan in a particular protein glycoside. The MALDI-TOF mass spectrometer is capable of analyzing even complex mixtures by producing complex, and non-complex spectra. This technique tolerates cleaners such as salts or buffers, allowing for quick and easy further sample preparation (Y. Zhang et al., 2021). Therefore, MALDI-TOF is one of the commonly used methods for glycan analysis.

1.2. Gut microbiota and Prebiotics

Microbiota means the entire community of microorganisms that colonize a particular area. The human gut microbioata includes most bacteria as well as other microorganisms such as fungi, archaea, viruses, and protozoans. The human gut microbiota contains about 100 trillion microorganisms. These microbes have important roles on human gut health. They perform different crucial tasks such as producing short chain fatty acids which are used as fuel for epithelial cells, producing vitamins, detoxifying toxins, maintaining of cholesterol metabolism, deconjugating bile, providing colonization resistance to pathogens, and regulation of gene expression. A healty human gut microbiota contains nearly 90% of phyla *Firmicutes* and *Bacteroidetes*. *Firmicutes* contains many genera which are usually include Lactobacillus, Bacillus, Enterococcus, Ruminicoccus and Clostridium. In adult gut, many bacterial species are non-sporing anaerobes for example Bacteroides spp. Bifidobacterium spp., Eubacterium spp., Clostridium spp., Lactobacillus spp., Fusobacterium spp (Swidsinski et al., 2005).

Microorganisms in the intestinal colon break down certain substrates from diet or endogenous secretions. These substrates used in colonic fermentation are usually starches and soluble dietary fibers. Carbohydrates such as oligosaccharides, non-absorbable sugars and sugar alcohols are substrates that are used for fermentation. Also aminoacids and proteins, bacterial secretions, lysis products, sloughed epithelial cells, and mucins are suitable for the gowth of these colonic bacterias. Many bacterial enzymes are degrade these substrates and produce intermediates. The intestinal bacterias ferment these intermediates to organic acids, histamine, carbon dioxide, and different neutral, acidic, and basic end products. Since these gut bacteria have metabolic activities, they can be called as beneficial. Their health-beneficial properties include immunoprotection, increase digestion and absorption, synthesis of vitamin, decrease the growth of pathogens, cholesterol reduction, and lowering of gas distension. Bifidobacteria and Lactobacilli are examples of beneficial bacteria in the gut. They can help for digesting lactose in lactose- intolerant individuals, decrease constipation and infantile diarrhea, help to resist infections and reduce inflammatory conditions in the gut. Both probiotics and prebiotics can be used to increase lactate-producing microbes. The probiotic approach promotes utilizing of living organisms in the diet. In another approach, it aims to increase the amount of bacteria that

improve health in the gut. These bacterias are selectively promoted by utilizing indigestible carbohydrates known as prebiotics.

The microbiome includes the complete genome of microorganisms in a particular environment. The human genome contains 23,000 genes, and the microbiome encodes about three million genes that produce thousands of metabolites that play a role in the health of the host. Human GI tract contain more than 1,000 diverse bacterial types, mostly in the colon. At birth, the human microbiota is nearly sterile and shaped by the maternal microbiome. Human gut microbioata plays important roles on detoxification of toxins, controlling cholesterol metabolism, deconjugation of bile, production of short chain fatty acids (SCFA), helping for colonization resistance to pathogens. However, the main aim of microbioata is growing and surviving.

Gut microbiota is dynamic and affected by many different factors such as age, genetics, environment, lifestyle, and especially diet. Twin studies revealed that although inherited components affect the gut microbiota, environmental factors such as diet and medications have much more important roles in the gut microbiota (Simões et al., 2013). One of the major external factors influencing the composition of the microbial community is dietary changes. Certain selected groups of bacteria are affected by changes in diet, such as prebiotics, high protein diet, probiotics, and fruits. When changes in the amount and type of indigestible carbohydrates present in the diet, it affects the production of metabolic products in the GI tract and bacterial population levels in feces. The relationship between dietary factors and gut microbiota is crucial for maintaining human health and homeostasis.

Gibson and Roberfroid defined prebiotics as "an indigestible food ingredient that beneficially affects the host by selectively stimulating the presence of one or a limited number of bacteria in the colon, thereby improving host properties" (Gibson and Roberfroid, 1995). Since food is the main factor controlling the intestinal microflora, it is possible to change the composition of the microflora through food. A prebiotic substrate is selectively utilized by beneficial components of the native intestinal flora, but does not develop potential pathogens such as toxin-producing clostridia, proteolytic bacterioids, and toxigenic Escherichia coli. In this way, a "healthier" microflora composition is achieved, in which Bifidobacteria and/or Lactobacilli predominate in the gut and exert possible health-promoting effects. At least three criteria are required for a food substrate to be classified as prebiotic: (1) the substrate must not be hydrolyzed or absorbed in the stomach or small

intestine. (2) It should be selective for beneficial commensal bacteria such as bifidobacteria in the colon. (3) fermentation of the substrate should cause luminal/systemic effects beneficial to the host. Recently, it has been found that *N*- or *O*-glycans which are separated from glycoproteins can be utilized by probiotic bacteria such as Bifidobacterium and/or Lactobacilli species, which are known to colonize the human gut with beneficial bacteria. Therefore, they should be investigated in detail. They can be isolated from glycoproteins of animals, plants, bacterias and fungi.

1.3. Mushrooms

Fungi are categorized as the "third kingdom," made up of organisms that fall outside of the classical dichotomy between animals and plants. Fungi play vital roles in many ecosystems as they are found all over the world. Fungi can have mutualist, pathogenic, and commensal roles in ecosystems. They are involved in connecting different micro and macro eukaryotic organisms or unicellular yeasts to complex fungi. Many fungi species have been found in extreme habitats such as volcanic, extremely cold and hot deserts, deep dead seas etc. Mainly 120 000 fungi species has been defined among existing 1.5-5 million species. It is hard to distinguish a certain species from other because of their tight connection via lichen, mycorrhizal link and cross-kingdom horizontal gene transfer between fungal species and other organisms.

The current taxonomy includes known fungi and divides them into nine major strains: Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Ascomycota, and Basidiomycota (Naranjo-Ortiz and Gabaldón, 2019). Mushrooms generally contain basidiomycetes and some of ascomycetes species. Although it is supposed that there are about 140 000 fungal species in the world, only 10% of them are known. It is thought that 5% of undiscovered or unknown mushroom species are beneficial to human health, indicating about 700 mushroom species. In addition, the number of mushrooms investigated among known species is very small.

Mushrooms can be branched into three parts: medicinal, cultivated, and wild mushrooms. Both wild and cultivated mushrooms are used by consumers due to their high nutritional qualities. In past decades, many mushroom species has been defined as edible and non-edible mushrooms. They have been used both in the food industry for their nutritional value and medical treatment purposes for their bioactive substances. Edible

mushrooms are consumed due to their flavour taste and also nutritional values by ancient people (Chang and Buswell, 1996). Historically; Romans and Chinese has been mentioned mushrooms as the power of strength and life. Chinese called them the elixir of life and Greeks called them as the food of Gods. Also, People in India, china and Iran used them for their religious activity. They are usually covered with branched hyphae and the hypas are divided. Their hypae specialized for reproduction. They reproduce by asexual reproduction using spores. Spores of Basidiomycetes mushrooms are produced outside of the mother cell. Spores are found inside a specialized cell which is called "ascus". A typical basidium generally found as a single club- shaped cell which has four stalks and each stalk is called "sterigmata". Spores are firstly formed inside of the sterigmata. The cell matures and then spores emerge from the sterigmata due to surface tension. Mushroom is inoculated under the growth conditions with spores, then their mycellium grows to produce fruiting bodies of the mushroom. These fruiting bodies are generally defined as edible part of the mushroom and consumed by consumers.

1.3.1. Nutritional Content of Edible mushrooms

It has been found that mushrooms contain approximately 15% to 30% crude protein by dry weight (Mdachi et al., 2004). Their protein contents vary according to species, varieties, and fruit bodies grown in different environments. According to the Food and Agriculture Organization (FAO) standard, the protein quality of the mushroom is higher than that of many plants. The in-vivo digestibility of mushroom proteins is between 73% and 76%, and this range is comparable to legumes, whose digestibility is around 70-80% (Dabbour and Takruri, 2002). The amino acid content and composition of mushrooms differ between mushroom species. In general, it has been reported that mushrooms contain about 30-50 g of essential aminoacids in 100 g protein content (Manzi et al., 1999). These aminoacids can be listed as histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Lysine, leucine, isoleucine, and tryptophan are other limiting amino acids in addition to methionine, which is a limited essential amino acid. Histidine and arginine, two semi-essential amino acids found in mushrooms, are crucial for growing children. Aspartic acid and glutamic acid are abundant as non-essential amino acids in mushrooms.

Both digestible and non-digestible carbohydrate contents of mushrooms differ between mushroom species, and the total amount is between 35% and 70% by dry weight

(J. H. Yang et al., 2001). Mushrooms contain low amounts of mannitol and glucose as digestible carbohydrates (Cheung, 2010). This means that mushrooms generally do not use carbohydrates as an energy source. Trehaloses, oligosaccharides, and non-starch polysaccharides (NSPs) like chitin, -glucans, and mannans are among the indigestible carbohydrates found in mushrooms. Mushrooms contain non-starchy polysaccharides that can be regarded as dietary fibers with known health benefits. The dietary fiber content of mushrooms differs according to the species, but essentially 100 g fruit body of a mushroom species is sufficient to meet 5% to 25% of an adult's daily fiber intake (Manzi et al., 2001). The dietary fibers of mushrooms are generally insoluble in water.

Numerous different minerals, including potassium, phosphorus, magnesium, zinc, selenium, and copper, are found in mushrooms. Mainly, cultured mushrooms contain various types of vitamins such as niacin, riboflavin (B2 vitamin), folates, and ergocalciferol which can be converted to vitamin D accompanying the existence of sunlight.

Mushrooms contain small amount of lipids that generally lower than 5% in dry weight. They contain low amount of linoleic acid as unsaturated fatty acid. This linoleic acid is the precursor of an aromatic compound named 1-octen-3-ol, that is noted to influence the flavor of the mushroom(J. H. Yang et al., 2002).

CHAPTER 2
PREVIOUS STUDIES

2.1. Health Beneficial Properties of Mushrooms

Mushrooms contain phenolic compounds as a secondary metabolite which show antioxidant activities. In a study, polysaccharides of *Jisongrong* mushroom has been found to have a good antioxidant and antitumor activity (Zhou and Chen, 2011). These polysaccharides were given to rats orally for 2 months and compared with control groups. It was demonstrated that these polysaccharides increased the activity of antioxidant enzyme in the blood and decreased lipid peroxidation products level. In a different study, the antioxidant activity of polysaccharides of three various edible mushroom species was investigated and it was establish that total phenolic and protein content influence the activity of the polysaccharides (Siu et al., 2014). Polysaccharides that lack of phenolic substances and protein had no considerable antioxidant activity.

Medicinal mushrooms exhibit anti-inflammatory properties that benefit human health. An anti-inflammatory component of the Cordyceps sinensis medicinal mushroom has been studied, and a peptide called cordymin was isolated from the mushroom (Qian et al., 2012). In a study, the effect of cordymin on cytokine level and total antioxidant activity was examined using in vivo and in vitro applications (J. Wang et al., 2012). After Cordymin treatment, it was discovered that levels of tumor necrosis factor-alpha, interleukin 1 beta, and total antioxidants all dropped. In addition, it was determined that the level of acetic acid, which causes abdominal contractions in mice, decreased depending on the dose of cordymin. Therefore, it was revealed that cordymin was a drug with a role in anti-inflammatory activity. In a separate study, the anti-inflammatory activity of polysaccharides belonging to the *Lentinus edodes* mushroom species was investigated to find out the effects of these polysaccharides on colitis-induced necroptosis by using in vitro and in vivo models (Nishitani et al., 2013). It has been concluded that depending on the doses of polysaccharide given, it reduces the level of colitis and also prevents necroptotic cell death.

Recently, it has been revealed that mushroom based polysaccharides play an important role in the activation and differentiation of phagocytes, the protection of immune factors and cytokines, the activation of lymphocytes and the regulation of many processes in the immune system (Motta et al., 2021). Mushroom polysaccharides are generally

composed of many β-glucan molecules and these glucans have important physiological activities. Mushroom polysaccharides contain high amounts of mannose and rhamnose, which play vital roles in improving immune functions, activating the immune system and stimulating the immune system.

Many studies has demonstrated that mushrooms have biologically active compounds that have effective inhibitory activities against breast cancer, hepatocellular carcinoma, uterine cervix cancer, pancreatic cancer, gastric cancer, and acute leukemia (Panda et al., 2022). In particular, mushroom-based polysaccharides are of great interest because they are biologically active substances that have immune-protective and anti-tumor roles. The polysaccharides of mushrooms contain high amounts of fucose, which has anticancer activity. In addition, polysaccharides of some mushroom species contain glucuronoxylomannan, which has antitumor activity and stimulates vascular endothelial cells. A study shows that, some gold nanoparticules(AuNPs) that isolated from polysacchides of *pholiota adiposa* mushroom have anticancer activity and are used in biomedical industry (Z. Yang et al., 2022). These nanoparticles can increase the production of NO and cytokines as well as macrophage cells in vitro. The study suggests that these new particles may indirectly play an anticancer role in liver carcinoma, as they improve immune system activity.

Mushrooms contain many bioactive compounds that have the potential to develop antibacterial, antiviral and antifungal activities. In a study, the antifungal and antiviral activity of *Auricularia auricular-judae*, an edible-medicinal mushroom, was investigated (Cai et al., 2015). It was found that the extracts of the mushroom had antimicrobial activity againist to some bacterial species such as *Escherichia coli* and *Staphylococcus aureus*. In another study, the antibacterial activity of a 44 kDa molecular weight protein isolated from the fruit body of *Clitocybe sinopica* was investigated (S. Zheng et al., 2010). The protein was found to have potential activity against *Agrobacterium rhizogenes*, *Agaricum tumefaciens* and *Xanthomonas oryzae*.

Mushroom polysaccharides such as chitin, hemicellulose, mannans, α- and β-glucans, galactans, and xylans are currently considered as potential prebiotic candidates. In one study, feeding mice with *Agaricus bisporus* resulted in rising microflora diversity and reduced potentially pathogenic (Clostridia) bacteria in the GI tract (Jayachandran et al., 2017). Ingestion of *A. bisporus* has been found to stimulate the activation of the innate

immune system, which produces inflammation and increases the composition of the intestinal flora and maintains gut health by protecting the GI tract from damage and pathogens (Giannenas et al., 2011). Another study found that *Ganoderma lucidium* (GL) reduced obesity and improved intestinal microflora in mice (Jayachandran et al., 2017). GL causes weight loss lowered insulin resistance, and decreased inflammation in mice fed a fat-rich diet. Ingestion of GL also reduced the level of intestinal dysbiosis, altered intestinal barriers and lowered endotoxemia. Therefore, GL can be considered a prebiotic that strengthens the GI microbiome to prevent intestinal dysbiosis and obesity-related diseases. In a separate study, *A. bisporus* mushroom was consumed by turkey chicks and it was investigated how the mushroom changed gut microbiome composition, performance, and morphology (Giannenas et al., 2011). It has been found that it grows lactic acid-producing bacteria and develops intestinal status.

2.2. Fungal Glycans

Fungal glycans are generally isolated from frutiting bodies and also mycellium, spore of edible and medicinal mushrooms. They are high molecular wighted polymers that consist of monosaccharides linked with glycosidic bond. These glycans have a unique and complex structure that gives them intense polymer properties. More than 200 fungal glycans have been isolated from 300 mushroom species in China and about 30 of them have significant pharmaceutical properties (Y. Zhang et al., 2021). Some fungal glycans that isolated from edible mushrooms can be listed as lentinan glycan from *Lentinus edodes, Polyporus* glycan from *Polyporus versicolor*, glycan from *Flammulina velutipes* and glycan of *Hericium erinaceus.* These glycans have many important roles on physiological processes such as anti-tumor, anti- viral, anti-oxidant and hypoglycemic effects (Ying and Hao, 2023).

Structure of fungal glycans are complex because of their high molecular weight, various monosaccharide composition and glycosidic linkages. Isolation of fungal glycans can be done by various methods and different glycan fractions can be obtained due to isolation method (D. Yang et al., 2019). These isolation methods include extraction of glycans using hot water, acid, alkali, enzyme, ultrasound and microwaves (W. Wang et al., 2022). Different type of glycans can be isolated according to using method but the most well-known fungal glycan types are β-glucans and mannans.

β- glucans are isolated from cell wall of mushrooms and have glycosidic bond-linked glucose (Timm et al., 2023). They are the most studied fungal glycans and are mainly characterized by the presence of β(1,6) linked branches originating from the β(1,3) backbone. Among the many polysaccharides of mushrooms, β-glucans are an important component of the use of mushrooms in cosmetics, as food additives or for medicinal purposes. β-glucans are natural molecules that have great therapeutic potential due to their immunomodulatory, antineoplastic, anti-inflammatory, antioxidant, anti-allergic, antibacterial, antifungal, and antiviral properties (Zhu et al., 2016).

Mannans are formed of α glycosidic bond-linked mannoses, especially enriched in α (1-3) and α (1-2) linked mannoses (D. Yang et al., 2019). Mannans are isolated from fungal cell wall. They are generally composed of short *O*-linked and *N*-linked mannans which are decorating glycoproteins. It has been reported that mannan has immunomodulatory effects in vivo by interacting with the mannose receptor and stimulating macrophages.

The health-promoting properties of fungal glycans are affected by some properties of glycans, such as the orientation and length of the construct and the presence or absence of side chains. Fungal glycans are isolated from many different fungal species and they have been shown to have potent anti-tumor and immunomodulatory activities (E. A. Murphy et al., 2010). Also, fungal glycans that bind to proteins or peptides have been found to have higher anti-cancer activities than free fungal glycans (Ooi and Liu, 2000). They reduce tumor progression by activating innate and adaptive immune system.

There are some edible mushroom glycans that are used as drugs in China approved by Chinese Food and Drug Administration (SFDA) such as lentinan from *L.Edodes* mushroom and *P.Umbellatus* glycans (Zhangrun Han, 2014).

Lentinan is isolated from fruiting body of an edible mushroom species which is called *L. Edodes*. Lentinan consists of β-glucan containing β-(1–3)-glucose backbones and branching with each of the five glucose units (1–6)-β-glucose. Lentinan-based drugs are produced in the form of capsules, tablets and injections. These drug products are generally used in the treatment of different types of cancer, such as lung, stomach and colorectal cancers in China (M. Zhang et al., 2019).

The main glycan of *P.Umbellatus* is β-glucan is isolated from mycelium of *P. Umbellatus* which is an edible mushroom species. The β-glucan of *P. umbellatus* include glucose in its (1-3) backbone and (1-6) side chains. Pharmaceutical products containing *P. umbellatus* glycan are produced in the form of capsules and injections. Some clinical studies have found that capsules are effective in the treatment of hepatitis B, and that injections can be used for decreasing recurrent bladder cancer (Guo et al., 2019). In addition, Polyporus glycans can be used alone or in combination with chemotherapy drugs in the treatment of various cancers such as stomach, liver, leukemia and cervical cancers.

The advantages of mushroom glycan-based drugs compared to commercially used protein or peptide compound-based drugs can be listed as follows; their small toxicity, wide range of therapeutic applications, low cost, less drug-resistant problems (D. Yang et al., 2019). However, there are some issues that need to be resolved in order to create safe glycan-based drugs for governments around the world to accept drug products. Some problems with fungal-glycan-based drugs are improving the quality and content of glycans, purifying highly complex glycans, understanding the pharmacodynamics of fungal glycan-based drugs, and performing reliable pharmacokinetic studies.

2.3. Edible Mushroom Species That Investigated in the Thesis

Although there are a wide variety of mushroom species in Turkey, systematic studies on mushrooms were started in the 1930s and only mushrooms growing on trees were examined in Turkey. More than 50 years later, scientists began investigating the variation and distribution of local macrofungal species. As a result of these studies, many macrofungal species were examined and 2158 taxa were recorded for the Turkish mycobiota. In 2009, a new mushroom species belonging to the genus *Marasmius*, named *Marasmius castaneophilus*, was discovered in Turkey (Işıloğlu et al., 2009). *M. castaneophilus* was called as chestnut mushroom due to it has a chestnut tree as the host (DOĞAN and Kurt, 2016). *M. castaneophilus* can be distinguished from other fungal species by its typical chestnut host, stem that turns red-brown with age and big basidiosporus.

Agaricus bisporus, or white button mushroom, is one of the most cultivated edible mushrooms in the world. *A. bisporus* is classified in taxonomy in the basidiomycota kingdom. Many studies have found that *A. bisporus* has antimicrobial, anticancer,

antioxidant, antitumor and immunomodulatory properties (Jeong et al., 2012). It has also been shown to be a good source of fiber, carbohydrates, protein, and low in fat and energy. Particularly the fruiting bodies of *A. bisporus* include high protein content. Each 100 grams of dry *A. bisporus* contains 19-38 grams of protein (Braaksma and Schaap, 1996). It contains essential, semi-essential and non-essential amino acids. It includes high amounts of lysine, alanine, glutamic acid, aspartic acid, serine, proline and low amounts of valine, methionine amino acids.

Pleurotus ostreatus, known as Oyster mushroom, is one of the most widely grown and well marketed edible mushroom species. It contains 20-15% protein, 13-24% fiber and 4-5% fat by dry weight. *P. ostreatus* includes 17 to 42 grams of protein per 100 grams of dried fruit body. The fruit bodies of *P. ostreatus* have been found to have health-promoting effects such as immunomodulatory, antioxidant, antitumor, anti-inflammatory, cholesterol-lowering, and potential prebiotic properties (Sarangi et al., 2006). Also, *P. ostreatus* proteoglycans have been found in its fruiting bodies. These proteoglycans consist of bioactive compounds such as arabinose, mannose, galactose, glucose and galacturonic acid, and their molar ratios are 1:0.9:1.7:5:0.6 (Xia et al., 2011).

Figure 1. Mushroom species that were examined in the thesis. *Pleurotus ostreatus*, *Agaricus bisporus*, and *Marasmius castaneophilus* mushroom species are shown, respectively.

2.4. Intestinal Dysbiosis and Disease Correlation

A stable microbiota of a healthy individual is considered normal microbiota. However, the disruption of hemostasis between the microbiota and the host is called dysbiosis. Permanent instability of the microbial community in the gut causes dysbiosis

and it is related to some diseases such as inflammatory bowel diseases (IBD), obesity, cancer and central nervous system diseases (Honda and Littman, 2012).

The gut microbiota influence the brain through gut-brain axes. Many experimental studies have revealed the regulatory effects of the gut microbiota on brain interactions (Sherman et al., 2015). Various neurological diseases, anxiety, depression, parkinsonism, Alzheimer's and multiple sclerosis are affected by the gut microbiota. Bacterial dysbiosis can cause signals for body defense to be synthesized and lead to CNS disease progression. Microbial strains in the gut have roles on synthesizing main neurotransmitters such as dopamine, norepinephrine, serotonin and histamine (Dicks, 2022). In addition, the vagus nerve has an important role in the communication of the gut with the brain, which affects mental health and cognition in the stress response. Signals of the gastrointestinal tract and gut microbiota are carried to the brain by vagus nerve fibers that are attached to various parts of the digestive tract, including the esophagus, liver, and pancreas (Han et al., 2022).

Many factors affect in the cancer progression such as genetics, lifestyle habits and diet. Today, many confirmations have been found that the the gut microbiome has crucial roles in cancer development. It has been found that intestinal dysbiosis can adversely affect the host's metabolism and immune functions, thereby promoting tumor proliferation (Asseri et al., 2023). Intestinal dysbiosis can trigger both local and distant tumors. Tumor progression has been found to increase by 20% in cases of intestinal dysbiosis (Bhatt et al., 2017). Kidney disorders are also correlated with intestinal dysbiosis. An imbalanced microbiome increases the synthesis of harmful metabolites such as uremic toxins and reduces the amount of SCFAs, which are the main mechanism of intestinal dysbiosis in kidney diseases.

CHAPTER 3
MATERIAL AND METHOD

3.1. Sample Collection and Preservation

Marasmius castaneophilus, Agaricus bisporus and *Pleurotus ostreatus* mushroom species were collected from a local market in Çanakkale, Turkey. Samples were preserved at 20 ºC.

3.2. Fungal Protein Isolation and Quantification

Approximately 3 g of sample from each mushroom species were smashed by pestle mortar. 300 µL dH_2O was added to each sample and samples were homogenized by using laboratory blender machine. Then, lysis buffer(1: 1 ratio) added and incubated on an ice bath for 1 hour. After incubation, samples were centrifuged at 4000 rpm for 10 min. After centrifugation, supertanant part, which contain dissolved proteins, is collected and cold ethanol was added at 1: 4 ratio for precipitating proteins at 20 ºC for overnight. After ethanol precipitation, samples were centrifuged at 400 rpm for 10 min. Pellet part which contain collapsed proteins is collected and dissolved in 300 µL dH_2O. Approximately 1: 3 of added dH_2O was removed by using vacuum evaporator to obtain more concentrated proteins.

Protein quantification was done by using a Qubit fluorometer machine. For every 2 µL sample; 1 µL of qubit dye and 197 µL of qubit buffer were added to the qubit tube and the tube was incubated for 15 min. Then, protein concentrations were measured.

SDS-PAGE gel electrophoresis BioRad system was used for visualizing fungal protein fractions. Protein bands were analyzed by laemmli procedure which is containing 12% separating gel (dH_2O, 40% Acrylamide, 1.5 M Tris- HCl PH: 8.8, 10% SDS, 10% Ammonium Persulfate, TEMED) and 4% stacking gel(dH_2O, 40% Acrylamide, 0.5 M Tris HCl PH: 6.8, 10% SDS, 10% Ammonium Persulfate, TEMED). SeeBlue™ Pre-stained Protein Standard ladder was used for determination of fungal proteins molecular weight. For 20 µL of protein from each sample, 1:1 v/v Laemmli buffer was added and samples were incubated at 95ºC for 5 min. Prepared samples were loaded to wells of SDS gel and Bolt™ MES SDS Running Buffer (Novex) was added for running the gel. After running the gel, the protein fractions were separated according to their molecular masses, and they were displayed in different bands on the gel according to the mass differences. For

visualizing protein bands, the gel was stained by Coomassie Brilliant Blue for 30 minutes at 55 rpm. A destaining solution (50% dH_2O, 40% Methanol, 10% Acetic acid) was added to remove the gel from Coomasie Brilliant Blue.

3.3. Recombinant PNGase F Enzyme Purification

In our previous study, the gene region that was responsible for PNGase F enzyme production was cloned into E.cloni bacterial cells and was preserved in ice. At this stage, some E.cloni bacterial cells from ice were transferred into a 200 mL bottle containing 250 mL of LB-kanamycin medium. The cap of the bottle was wrapped with aluminum foil and left to incubate in a shaking incubator at 37 ºC, 160 rpm. About 2 mL of the sample was taken from the bottle and OD measurement was taken at 600 nm wavelength in the spectrophotometer device. The measurements were repeated until the OD600 nm value reaches the range of 0.5-0.7. When the value in the desired OD range was reached, 0.2% Rhamnose was added and incubated overnight in a shaking incubator at 24 ºC, 1600 rpm. After this incubation, the growing bacterial cultures were transferred to 50 mL falcon tubes and centrifuged at 400 rpm, +4 ºC for 15 minutes.

After centrifugation, the pelleted part was collected and used for the lysis process and the pelleted part was incubated for 1 hour at -80 ºC. Then, it was taken to room temperature and dissolved with 5 mL of dH_20. It was centrifuged at 4000 rpm, +4 ºC for 15 minutes, and the pelleted part was collected. 6300 µL of lysis buffer and 63 µL of protease inhibitor were thawed by pipetting into the pelleted part and incubated for 30 minutes in an ice bath. The falcon tube containing the samples was placed in a beaker containing ice and placed in the sonication device. Cell fragmentation was ensured with 10-second pulses for 10 minutes in the sonication device. Then, it was centrifuged at 4000 rpm, +4 ºC for 5 minutes. The supernatant part was transferred to another falcon tube. 100 µL of the sample was taken and it was labeled as lysis and transferred to an Eppendorf tube for use in the SDS-PAGE analysis.

The batch method was applied for enzyme purification. An appropriate amount of Ni-NTA resin was removed from the buffer solution in which it was kept. For this process, 1 mL of Ni-NTA resin was added to a 50 mL falcon tube and centrifuged at 700x g for 2 minutes. After centrifugation, the pelleted part was collected and a 1:1 equilibration buffer

was added. Then, it was centrifuged at 700x g for 2 minutes. The supernatant part was discarded and by this way resin was removed from the buffer solution.

Protein samples were added in a 1:1 ratio to the Ni-NTA resin solution. Equilibration buffer was added to the resin-protein solution at a ratio of 1:1 and incubated for 30 minutes in a shaking incubator. It was centrifuged at 700x g for 2 minutes and the pelleted part was collected. Wash buffer at a 1:2 ratio added on the resin-protein portion that remained in the pellet and centrifuged at 700x g for 2 minutes. This process was repeated 10 times. Elution buffer at a 1:2 ratio was added to the pellet that was formed as a result of the 10th washing process. Then it was centrifuged at 700x g for 2 minutes and the resulting supernatant part was collected for further processing. The elution process was repeated 3 times. 100 µL of each supernatant that was formed as a result of each process was taken and labeled as E1, E2, E3 and used in the SDS-PAGE analysis.

3.3.1. Visualization of PNGase F Enzyme

SDS-PAGE gel electrophoresis BioRad system was used for visualizing the PNGase F Enzyme. For the preparation of SDS gels, 12% separating gel (40% acrylamide, 10% SDS, 1.5 M Tris PH 8.8, 10% Ammonium persulfate, TEMED, dH2O) and 4% stacking gel (40% acrylamide, 1M Tris pH 6.8, 10% SDS , 10% Ammonium persulfate, TEMED, dH2O) was prepared. Prepared gels were poured into the SDS mechanism and SDS combs were placed. Laemmli buffer (1:1 v/v) was added to each of the 20 µL lysis(E1, E2, E3) protein samples and incubated at 95 ºC for 5 minutes. 5 µL of SeeBlue™ Pre-stained Protein Standard ladder was used and 30 µL of each sample was loaded to SDS wells. Bolt™ MES SDS Running Buffer (Novex) was used. The gel was run by applying 200 volts of electric current. After running the gel, a protein band appeared at the region of the enzyme's molecular mass. To make this band visible, the gel was stained with Coomassie Brilliant Blue and incubated in a 55 rpm, 30 min shaking incubator. Then, a destaining solution was used to remove the Coomasie brilliant blue from the gel.

3.4. Deglycosylation of Fungal Glycoproteins

The recombinant PNGase F enzyme was used to perform N-glycan separation from fungal glycoproteins. 1 µl of glycoprotein denaturation buffer (10x) was added to 1-20 µg of fungal glycoproteins and dH_2O added to make the total volume 10 µl. They were incubated at 100 ºC, for 10 minutes for denaturation of glycoproteins. After incubation, the

total reaction volume was 20 µl; 2 µl of glycol buffer 2 (10x), 2 µl of 10% NP-40, dH_2O, and 1 µl of PNGase F was added. 37 ºC, 1-hour incubation was carried out.

3.5. Measurement of Glycan Concentration By Fenol Sulfuric Acid Assay

The phenol-sulfuric acid test was used to determine the total carbohydrates in the sample to confirm that protein deglycosylation had taken place. It is a fast and cheap method that shows the concentrations of all monomeric, oligomeric and polymeric carbohydrate samples by using a glucose standard. In this method, concentrated sulfuric acid breaks down any polysaccharide, oligosaccharide and disaccharide into monosaccharides. Pentoses (5-carbon compounds) are then dehydrated to furfural and hexoses (6-carbon compounds) are converted to hydroxymethyl furfural. These compounds then react with phenol to produce a yellow-gold color. For products high in hexose sugars, glucose is commonly used to construct the standard curve and absorption is measured at 485 nm.

a. For the preparation of glucose standards, 1 mg/mL glucose solution (2 mg glucose dissolved in 2 mL dH_2O) was prepared and serial dilution was made to finally give 1000, 800, 600, 400, 200 and 100 mg/mL glucose standards total volumes of 0.1 mL obtained. 25 µL of dH_2O was used as blank. All samples including blank, standards, control were prepared in duplicate.

b. 25 µL of each standard and sample was placed in 96 plate wells. 25 µL of 5% phenol solution was added to the standards and samples. Then, 125 µL of concentrated sulfuric acid was added. Samples and standards were then incubated at 35 rpm for 1 hour in the dark. After incubation, OD was measured at 485 nm and a standard curve was established.

3.6. Characterization of Released *N*- glycans by Using MALDI- TOF Mass Spectrometer

N-glycans was labeled with 2-aminobenzoic acid (2-AA). After mixing 50 µL of glycan solution with 25 µL of 2-aminobenzoic acid and 25 µL of 2-picoline borane, this mixture was incubated at 65 ºC for 2 hours. Pipette tips containing cotton were used for the purification of glycans. For this purpose, cotton fibers were placed on 100 µL pipette tips. Samples subjected to ethyl esterification were then used as the loading solution. The prepared pipette tips were washed with 100 µL of dH_2O and 100 µL of 85% acetonitrile,

respectively. The prepared loading solution was passed through these cotton-containing pipette tips at least 20 times. After this step, the pipette tips were washed 3 times with 85/14/1, v/v/v, acetonitrile/water/trifluoroacetic acid solution and 3 times with 85% acetonitrile to remove salt and other solvent residues. Elution was done with 10 µL of dH_2O. For MALDI- TOF-MS analysis, 1 µL of the elution solution was dropped onto the MTP 384 Anchor plate and dried. After this step, 1 µL of 5 mg Super DHB matrix solution (w/w, 9/1, 2-5 dihydroxy benzoic acid / 2-hydroxy-5-methoxy benzoic) in 1/1, (v/v) acetonitrile was prepared. At this point, 1 mM NaOH was added. After crystallization was occurred, analysis by MALDI mass spectrometry was performed with a Bruker rapid flex MALDI Tissue typer. The analysis was performed by collecting at least 10000 laser pulses in reflectron mode in positive ionization. Bruker rapid flex MALDI Tissuetyper TM mass spectrometry calibration was performed with a peptide mixture before each analysis. Mass spectra in the 1000-4000 mass range was obtained by applying 25 kV acceleration voltage. Analysis of 2-AA-labeled N-glycan structures was evaluated in negative ionization mode using the method described above using the DHB (5 mg/mL, prepared in water) matrix.

3.7. Prebiotic Activity Tests of *N*- Glycans

Bifidobacterium infantis, *Bifidobacterium kashiwanohense*, *Bifidobacterium bifidum*, *Bifidobacterium bohemicum*, *Lactobacillus rhamnosus*, *Lactobacillus brevis* and *Lactobacillus bulgaricus* are the bacteria to be used in this study. *N*-glycans were used as carbon sources in the environment where these bacterial species are found. Bifidobacterium species were grown under anaerobic conditions at 37 °C by adding different concentrations of *N*-glycans (0.5%, 1%, 2%) to an RPM medium without cysteine. Lactobacillus species were incubated at 37 °C in an MRS broth medium to which mushroom glycans (1%) are added under aerobic conditions. In addition, an MRS medium without any carbon source was used as a negative control, and a medium containing 1% glucose was used as a positive control. Bacterial growth was monitored by measuring optical density (OD 600) by using a spectrophotometer instrument.

CHAPTER 4
RESEARCH FINDINGS

4.1. Visualization and Quantification of Proteins of Different Mushroom Species

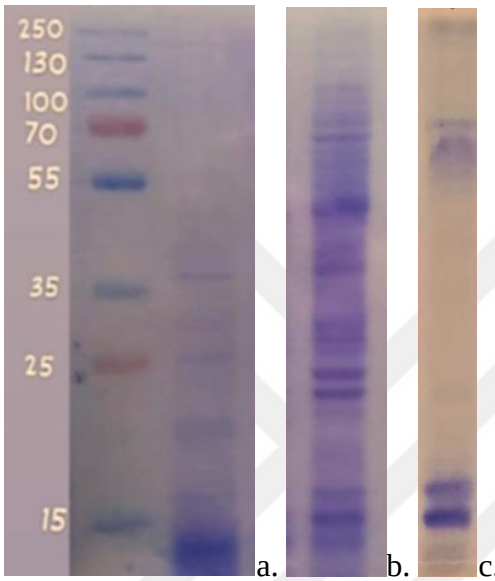

Figure 2. Visualization of mushroom protein fractions. SDS- PAGE results for (a) *Pleurotus ostreatus*,(b) *Agaricus bisporus* and (c) *Marasmius castaneophilus* fungal proteins are shown. Ladder shows different molecular mass bands from 15 to 250 kDa.

Table 1

Quantification of mushroom proteins.

Mushroom Species	Qubit Measurement(mg/ mL)
Pleurotus ostreatus	0.742
Agaricus bisporus	0.942
Marasmius castaneophilus	1.16

Qubit fluorometer 3.0 measurement results for proteins of *Pleurotus ostreatus, Agaricus bisporus* and *Marasmius castaneophilus* mushroom species are shown. It can be shown that *M. castaneophilus* has the highest protein content, while *P. ostreatus* has the lowest.

4.2. Visualization of PNGase F Enzyme by SDS-PAGE analysis

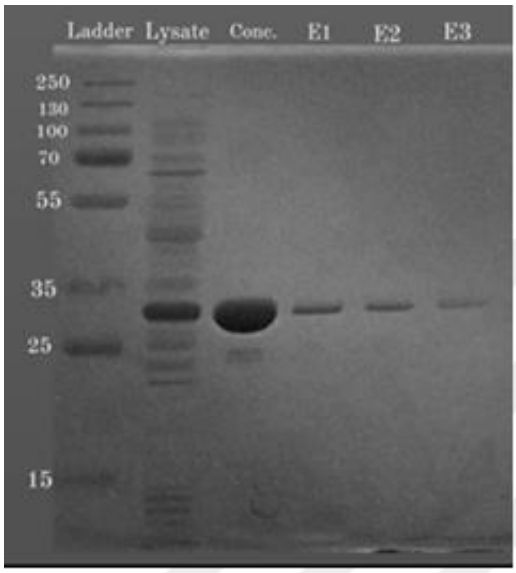

Figure 3. Visualization of Recombinant PNGase F enzyme. The SDS-PGAGE analysis results for lysis, concentrate, E1, E2 and E3 concentrations of recombinant PNGase F enzyme is shown. In lysate sample, the whole protein bands of the E.cloni bacteria are exist and an intense band can be shown about 34 kda which indicate the molecular mass of PNGase F enzyme. A huge and dense protein band of PNGase F enzyme is shown in the concentrated sample. E1, E2 and E3 samples contain a protein band of the purified enzyme. It can be revealed that a highly pure recombinant PNGase F enzyme is produced.

4.3. Determination of *N*- Glycan Concentrations of Mushrooms

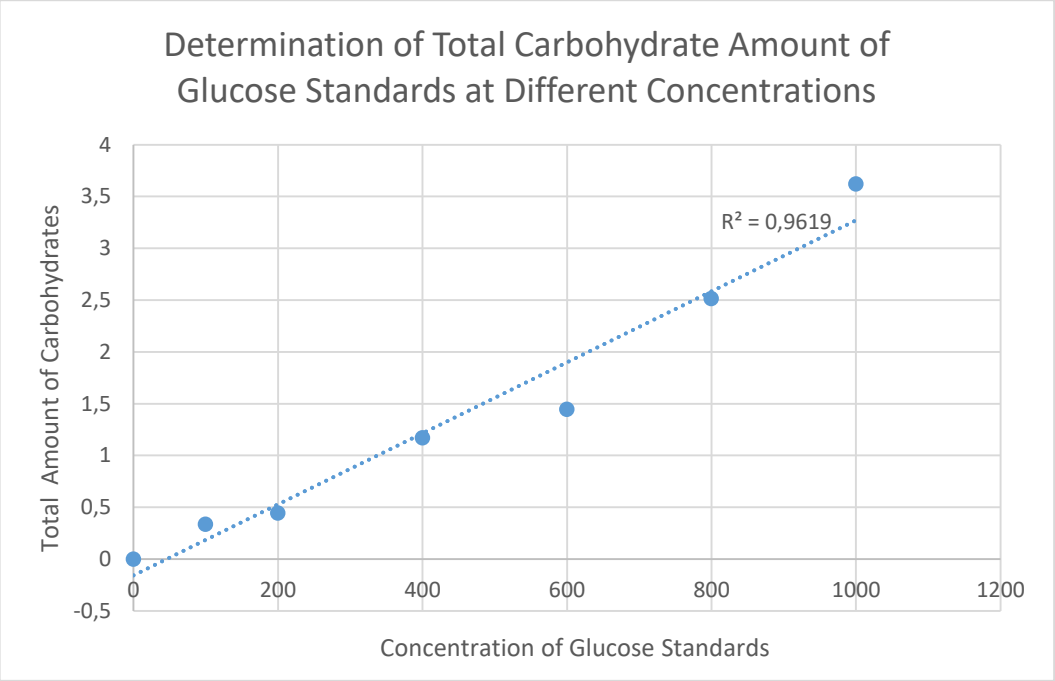

Figure 4. Determination of the total carbohydrate amount of glucose standards. The determination of the total carbohydrate amount of glucose standards at concentrations of 100, 800, 600, 400, 200, 100 mg/mL has been demonstrated by applying the phenol sulfuric acid assay.

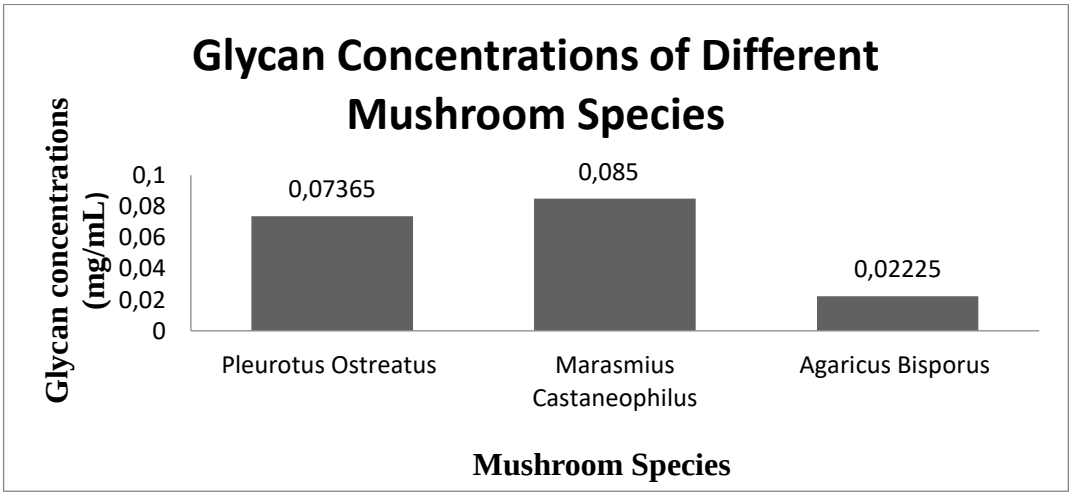

Figure 5. Glycan concentrations of Mushrooms. The phenol sulfuric acid test results of glycan concentrations of the mushroom species *Pleurotus ostreatus*, *Marasmius castaneophilus* and *Agaricus bisporus* are shown. *M. castaneophilus* has the highest concentration of *N*- glycans, while *A. bisporus* has the lowest.

4.4. Characterization of Mushroom *N*- Glycans BY MALDI-TOF MS

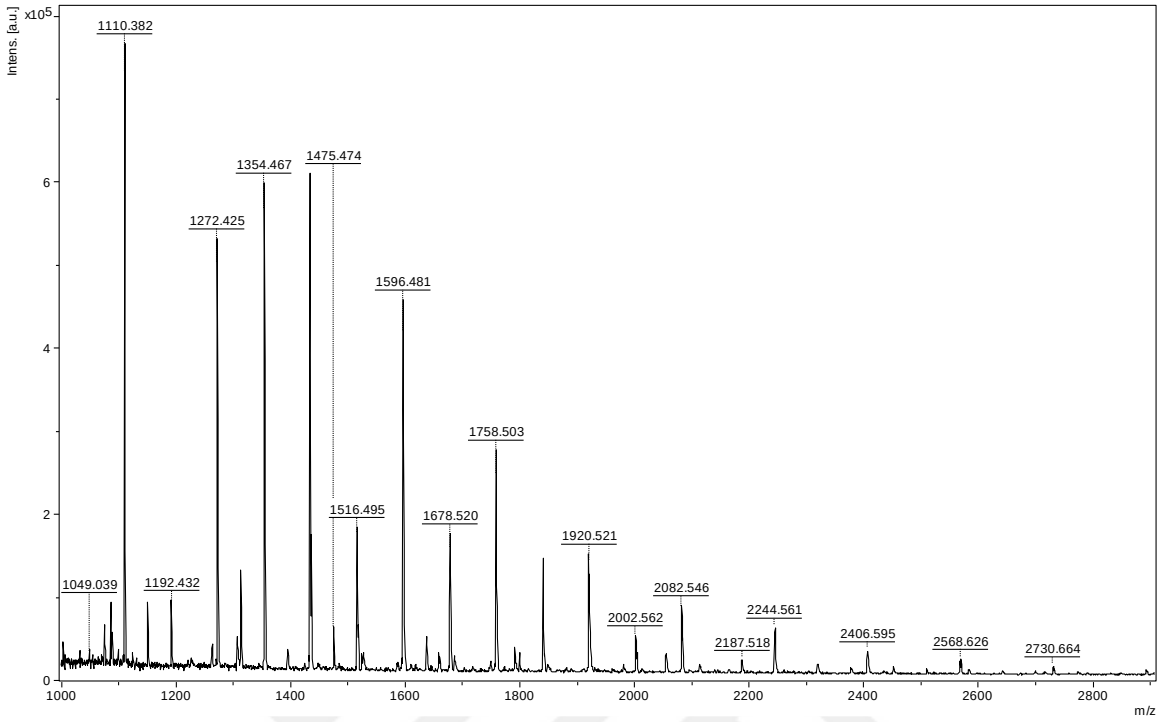

Figure 6. Characterization of *Pleurotus ostreatus N*-glycans. Mass spectra of *N*-glycan structures that obtained as a result of cleavage of glycoproteins from the *Pleurotus ostreatus* mushroom species by using PNGase F enzyme are shown. It shows that glycan structures with different densities and masses have been obtained.

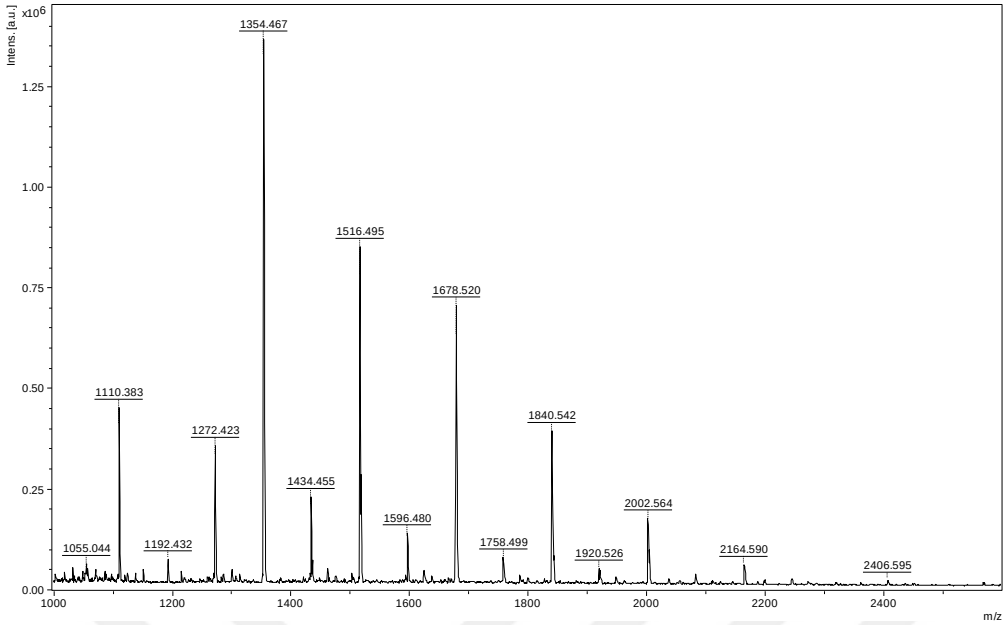

Figure 7. Characterization of *M. castaneophilus N*- glycans. Characterization of *N*-glycan structures obtained by cleavage of *Marasmius castaneophilus* mushroom glycoproteins with recombinant PNGase F enzyme by MALDI-TOF mass spectrometry analysis method is shown.

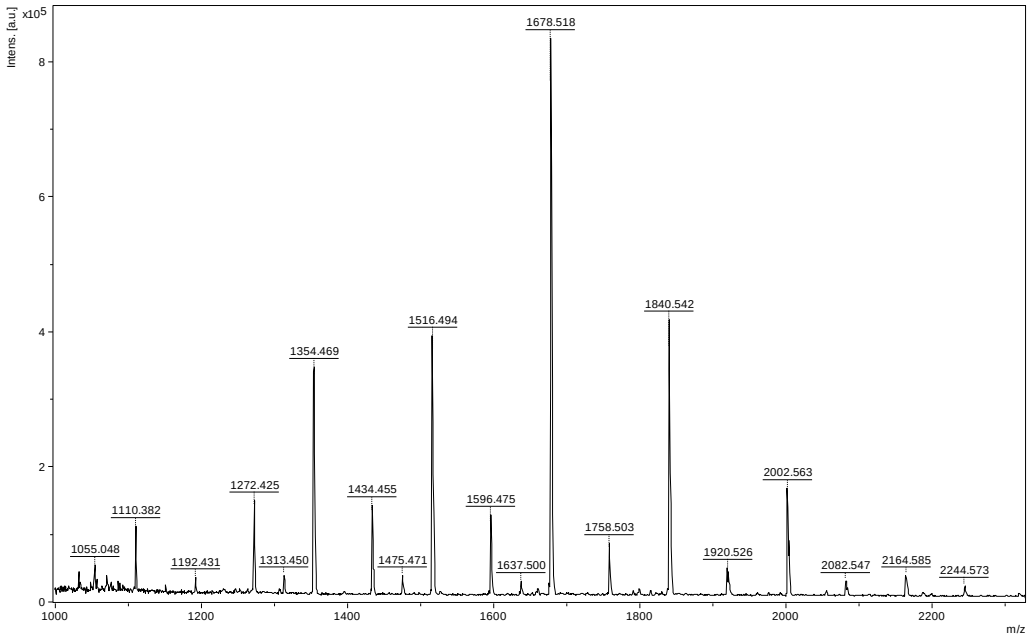

Figure 8. *N*-glycan characterization of *A. bisporus*. Mass spectra of *N*-glycan structures separated by PNGase enzyme from glycoproteins of *Agaricus bisporus* mushroom species are shown.

4.5. Testing Prebiotic Activity of Mushroom *N*-glycans

Lactobacillus rhamnosus, *Lactobacillus brevis* and *Lactobacillus bulgaricus* are the aerobic bacteria used in this thesis. *B. infantis*, *B. kashiwanohonse*, *B. bohemicum* and *B. bifidum* are anaerobic bacteria used in this thesis. *N*-glycans, which were separated from the mushroom glycoproteins can be used as carbon sources for these bacterial species.

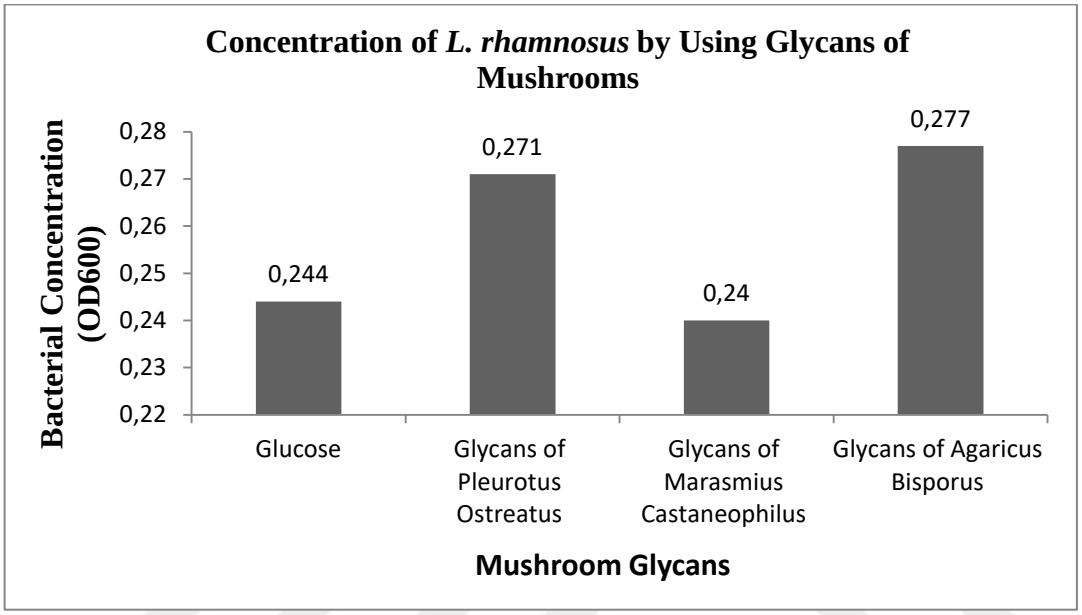

Figure 9. The use of mushroom glycans as prebiotics by *L. rhamnosus* bacteria. The concentration of *L. rhamnosus* bacteria in the medium with glycans of the mushroom species of *Pleurotus ostreatus*, *Marasmius castaneophilus* and *Agaricus bisporus* were measured as 0.271, 0271 and 0.277 mg/mL, respectively. Since bacterial growth was observed in all three environments, it can be said that glycans of all three mushroom species have a prebiotic effect on *L. rhamnosus* bacteria. *L. rhamnosus* bacterial concentration is highest in the presence of *A. bisporus* glycans. It can be said that the bacteria use the glycans of *A. bisporus*, which is a carbon source, very effectively. A medium containing 1% glucose with *L. rhamnosus* bacteria was used as a positive control. It can be seen that bacterial concentration is higher in the environments of *A. bisporus* and *P. ostreatus* glycans exist in the environment of glucose present. The concentration of *L. rhamnosus* in the presence of glycans of *M. castaneophilus* is the same as in the presence of glucose. It can be revealed that glycans of *P. ostreatus* and *A. bisporus* are very good prebiotic sources for *L. rhamnosus* probiotic bacteria. In addition, the glycans of *M. castaneophilus* can be used as a prebiotic source for this bacterium.

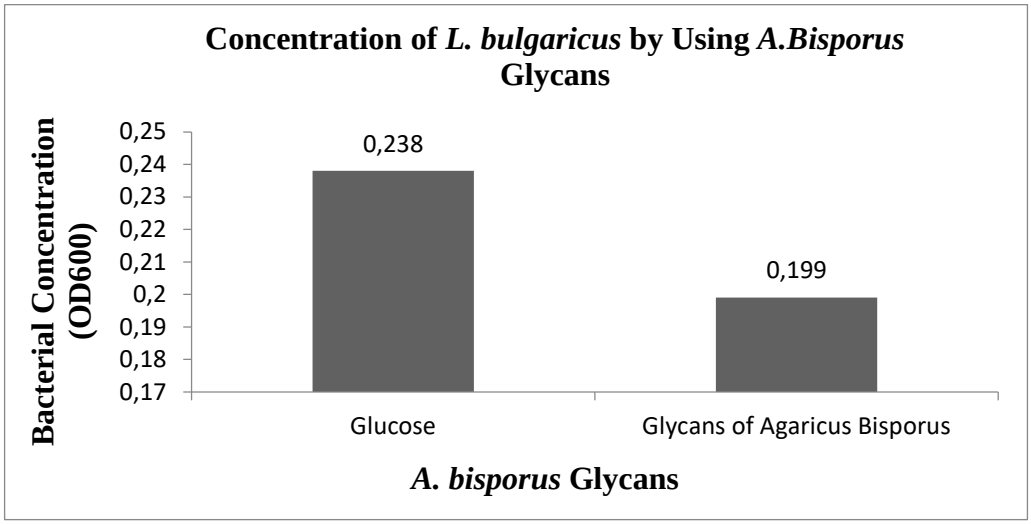

Figure 10. The use of *A. bisporus* glycans as prebiotics by *L. bulgaricus* bacteria. The concentration of *L. bulgaricus* bacterial species in the environment of *Pleurotus ostreatus*, *Marasmius castaneophilus* and *Agaricus bisporus* glycans was measured and it was determined that it only grew in the environment of *Agaricus bisporus* glycans. *L. bulgaricus* can grow in the environment of *N*- glycans of *A. bisporus* exist. However, the bacteria cannot grow in the presence of *M. castaneophilus* and *A. bisporus* glycans. The reason why *L. bulgaricus* does not grow in environments with other glycans may be the absence of *L. bulgaricus* carriers for these glycans.

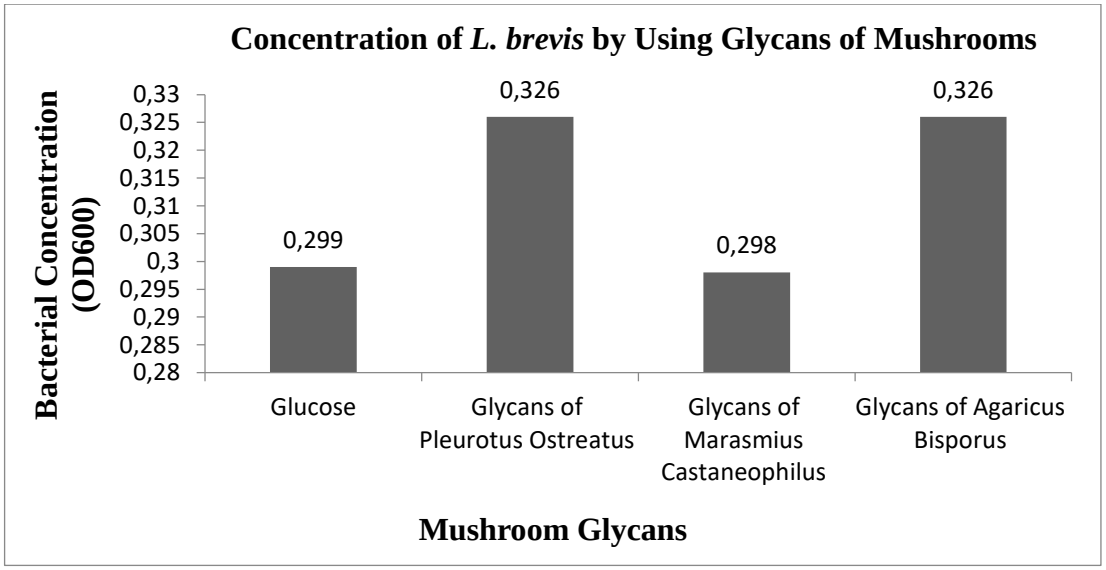

Figure 11. The use of mushroom glycans as prebiotics by *L. brevis* bacteria. The concentration of *L. brevis* bacteria in glucose media was measured as positive control. Glycans of *Pleurotus ostreatus*, *Marasmius castaneophilus* and *Agaricus bisporus*, have been shown to have a prebiotic effect on *L. brevis* bacteria. The *L. brevis* concentration is

0.326 mg/mL and is the same in the presence of *P. ostreatus* glycans and in the presence of *A. bisporus* glycans. It is shown that *P. ostreatus* and *A. bisporus* glycans are very good prebiotic sources with higher potency for *L. brevis* probiotic bacteria than the positive control. The bacterial concentration of *L. brevis* in the positive control and in the medium with the glycans of *M. castaneophilus* is very close. Therefore, these glycans can also be used as prebiotic sources for *L. brevis* probiotic bacteria.

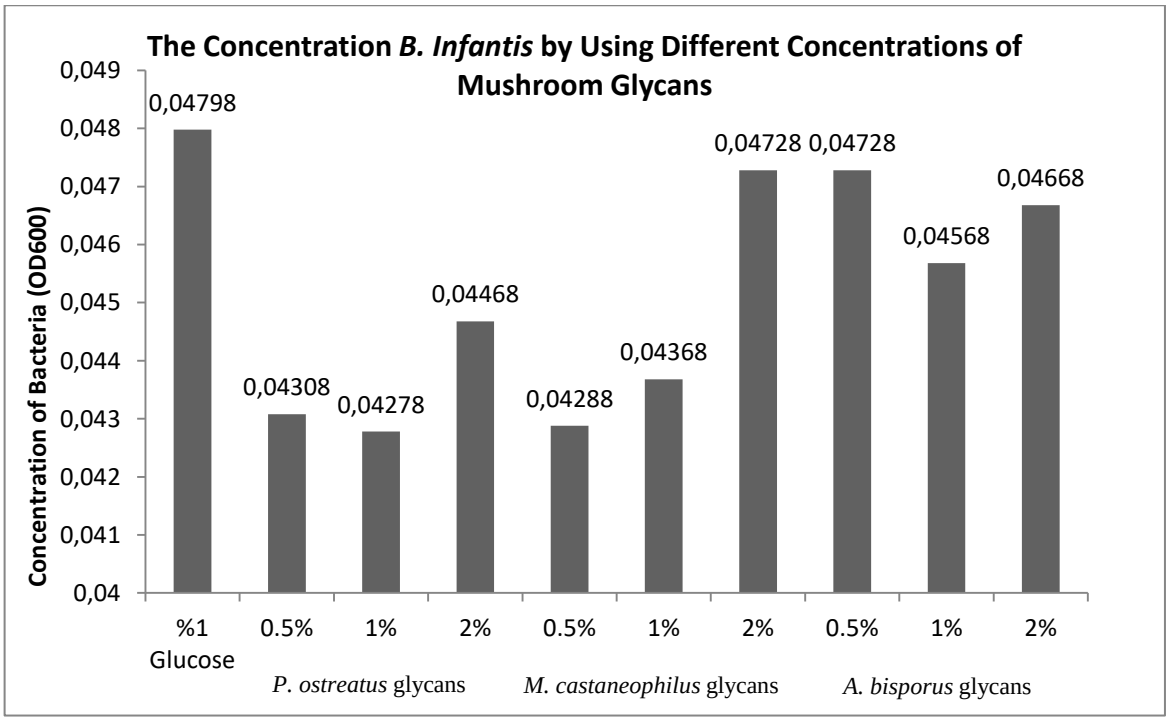

Figure 12. *B. infantis* bacterial growth in the presence of mushroom glycans at different concentrations. *B. infantis* growth in 1% glucose-containing media was used as the positive control and the bacteria grows mostly in this media as 0.04798 mg/mL. It is seen that the concentration of *B. infantis* is in the highest value at 0,04728 mg/mL both in 2% of *M. castaneophilus* glycan-containing environment and in the environment where 0.5% *A. bisporus* glycans are present. It can be revealed that *B. infantis* can effectively use these glycans as a carbon source and these glycans are very good prebiotics for this bacteria. In addition, the growth of *B. infantis* is measured at relatively high values in all environments containing mushroom glycans. It can be said that the glycans of *A. bisporus*, *M. castaneophilus* and *P. ostreatus* are used as carbon sources by *B. infantis*. When comparing the bacterial growth between different concentrated *P. ostreatus* glycans, it can be found that the *B. infantis* concentration has the highest value of 0.04468 mg/mL in the presence of 2% concentrated *P. ostreatus* glycans. It can be revealed that *B. infantis* effectively uses

2% *P. ostreatus* glycans instead of other concentrated *P. ostreatus* glycans as a carbon source. *B. infantis* concentration in the environment that contains 2% of *M. castaneophilus* glycans has the highest OD value at 0.04728 mg/mL when compared with other *M. castaneophilus* glycans. It has been shown that when the *M. castaneophilus* glycan concentration increases, the growth of *B. infantis* bacteria also increases. This may be because these bacteria use highly concentrated glycans of *M. castaneophilus.* The bacterial density of *B. infantis* has the highest value at 0.04728 mg/mL in the environment with 0.5% concentrated *A. bisporus* glycan when compared with other concentrations of *A. bisporus* glycans. It can be revealed that low concentration of *A. bisporus* glycans are better carbon sources for *B. infantis* than other concentrated *A. bisporus* glycans.

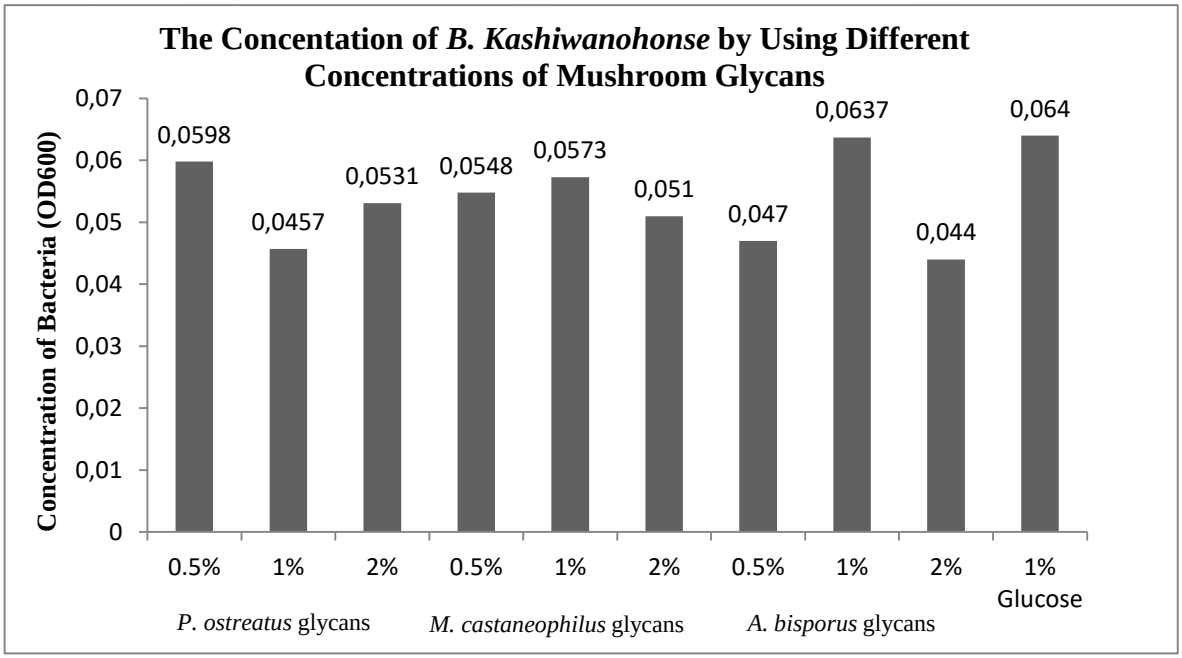

Figure 13. *B. kashiwanohonse* growth in the presence of mushroom glycans at different concentrations**.** *B. kashiwanohonse* concentration in the medium containing 1% glucose is shown as the positive control with the highest value of 0.064 mg/mL. It is because the bacteria use glucose as a better carbon source than mushroom glycans. In addition, it is seen that the concentration of *B. kashiwanohonse* in 1% concentrated *A. bisporus* glycan medium is 0.0637 and this value is very close to the positive control. It can be revealed that 1% concentrated *A. bisporus* glycans are used carbon sources as good as glucose. *B. kashiwanohonse* concentration has the highest value at 0.0598 mg/mL in the presence of 0.5% *P. ostreatus* glycans when compared to other environments with concentrated *N*-glycans of *P. ostreatus*. It can be said that 0.5% concentrated *P. ostreatus* glycans have

higher prebiotic activity on *B. kashiwanohonse* than other concentrated *P. ostreatus* glycans. The Figure shows that 1% concentrated *N*-glycans of *M. castaneophilus* have higher prebiotic activity on *B. kashiwanohonse* bacteria. Because the bacterial concentration in 1% concentrated glycans of *M. castaneophilus* has been shown to be 0.0573 mg/mL which value is higher than other *N*-glycan concentrations of *M. castaneophilu*s. It is seen that the growth of *B. kashiwanohonse* in the environment with 1% concentrated *N*-glycans of *A. bisporus* is higher than in other environments with *A. bisporus* glycans. It can be revealed that the prebiotic activity of 1% concentrated *A. bisporus* glycans on *B. kashiwanohonse* bacteria is very high.

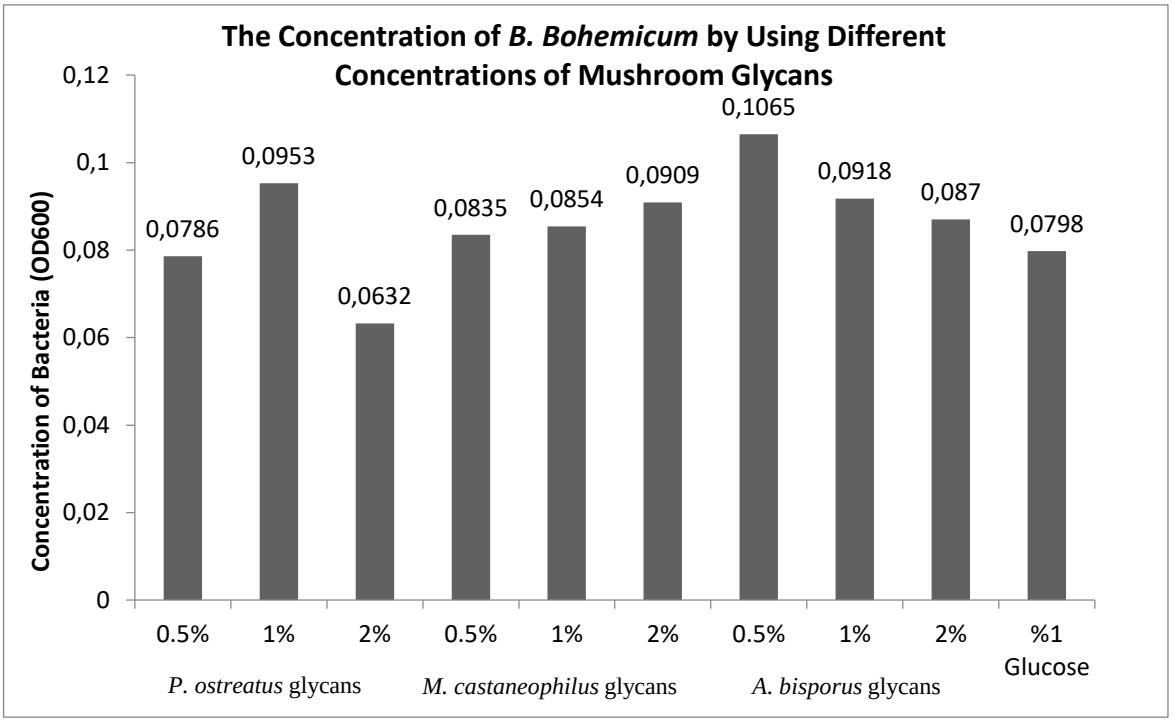

Figure 14. *B. bohemicum* growth in the presence of mushroom glycans at different concentrations. Medium containing 1% concentrated glucose is used as a positive control and the bacterial concentration in this medium is measured as 0.0798 mg/mL. When examining the Figure, bacterial density was measured higher in the presence of mushroom glycans than in the positive control, except for 0.5% and 2% concentrated *P. ostreatus* glycans, where bacterial growth was measured lower than the positive control. In general, it can be said that the concentration of *B. bohemicum* bacteria is very high in environments where mushroom *N*-glycans are present. It can be demonstrated that fungal glycans are considered by *B. bohemicum* as very good carbon sources, even better than glucose. The bacterial growth is very high in the environment that 1% concentrated glycans of *P.*

Ostreatus when compared with other environments with different concentrations of *P. ostreatus* glycans. It can be found that *B. bohemicum* uses 1% concentrated *N*-glycans of *P. ostreatus* very efficiently as a carbon source. It has been shown that when *M. castaneophilus N*-glycan concentration increases, bacterial growth also increases. This is because this bacterial strain uses the highly concentrated *N*-glycans of *M. castaneophilus*. Therefore, concentrated *N*-glycans of *M. castaneophilus* are very good prebiotic sources for *B. bohemicum* bacteria. It has been shown that bacterial growth is reduced when the concentrations of *A. bisporus* glycans are increased. It can be revealed that low concentrations of *A. bisporus* glycans are effective prebiotics for *B. bohemicum*.

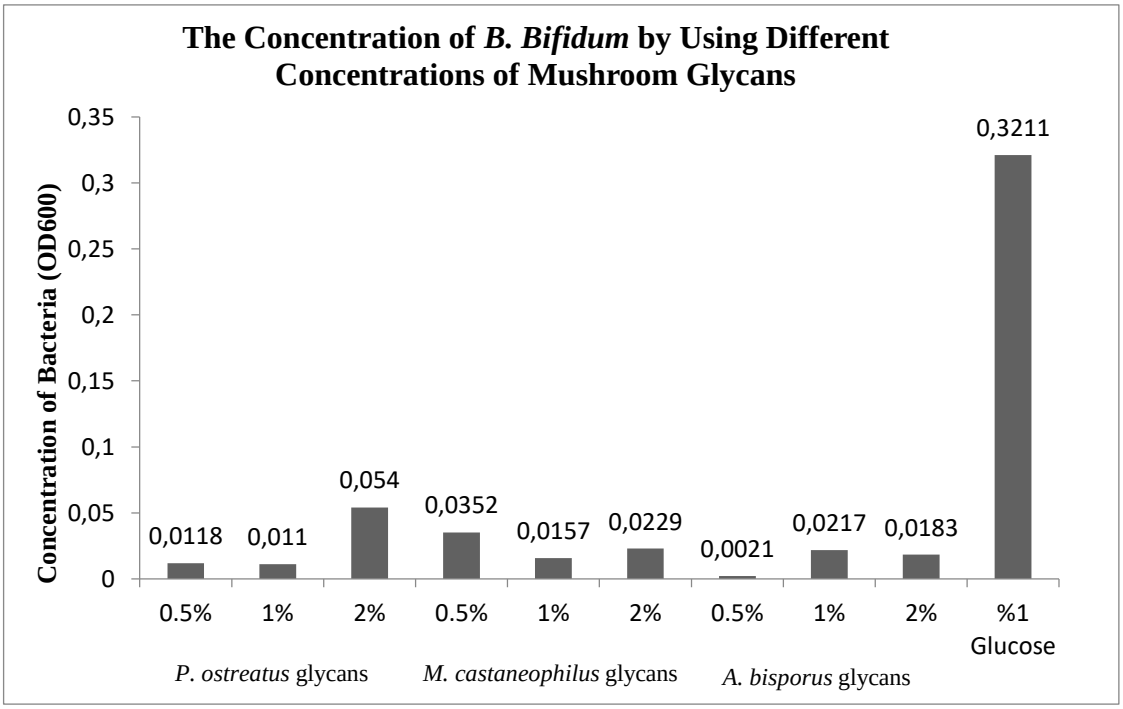

Figure 15. *B. bifidum* growth in the presence of mushroom glycans at different concentrations. The 1% glucose-containing media is shown as positive control and the bacterial concentration is measured as 0.3211 mg/mL in this medium. In general, bacterial concentrations have been measured in very low amounts in environments where mushroom glycans are present. The reasons may be due to the bacterial utilization of fungal glycans being too low efficiency or the concentration of fungal glycans being low for bacteria to use these glycans as a carbon source. The bacterial growth have been shown in the environment where 2% concentrated *P. ostreatus* glycans are present with a notable value. It can be revealed that 2% concentrated *P. ostreatus* glycans are better prebiotic sources for bacteria compared to other mushroom glycans.

CHAPTER 5

RESULT AND RECOMMENDATIONS

The fact that mushrooms can be produced easily and cheaply, the pH and thermal stability of their proteins are high, and that they can be grown even in areas where industrial wastes are found have attracted the attention of both the food industry and the scientific community. Mushroom proteins have attracted great interest of researchers because of their high protein content, which can be a good alternative to meat proteins. The protein content of mushrooms depends on their species. Therefore, protein fractions of different mushroom species are needed to be investigated in detail. In this thesis, protein quantification and profile of three different mushroom species was examined. First of all, proteins of mushrooms have been isolated by physical and chemical disruption methods. Isolated mushroom proteins were visualized by SDS-PAGE analysis method. Intense protein bands of *P. ostreatus* have been found around 25, 30 kDa (Figure 2). Protein bands between 10 and 190 kDa belong to lectins, which are glycoproteins that can bind to many carbohydrate moieties. Protein profile of the three mushroom species are distinct from each other. For example, while the protein band in *A. bisporus* mushroom has an intense density of about 47 kDa, this band is not shown in *M. castaneophilus* and it is very low in *P. ostreatus* mushroom. It can be revealed that protein bands of the mushrooms have heterogenity between species.

It is known that most of proteins that are found in nature in *N*- linked glycosylated form. These *N*- glycans are needed to be removed from attached proteins to obtain free glycans. Therefore, PNGase F enzyme which is a bacterial enzyme that cut all types of *N*-linked glycans from attached proteins is purified from E.cloni bacterias. Rhamnose was used to induce the bacteria to produce high amount of proteins. Bacterial cells were disrupted by chemicals and proteins were separated according to their mass through centrifugation. The whole isolated proteins were visualized by SDS-PAGE analysis (Figure 3.). A Ni-NTA resin-containing solution was used to separate the enzyme from other proteins, where the resin specifically binds to the enzyme. To obtain a pure enzyme; wash and elution buffers was used to remove impurities. A recombinant highly pure enzyme enzyme was obtained and visualized by SDS-PAGE method (Figure 3). This enzyme was used to remove *N*-linked glycans from mushroom glycoproteins, creating optimum conditions for the enzyme to work efficiently. The concentration of mushroom glycans were measured by phenol- sulfuric acid assay (Figure 5). MALDI-TOF MS was used for

the characterization of the obtained fungal *N*-glycans. It has been found that *P. ostreatus* glycans contain 2'-fucosyllactose and *N*-Acetyl galactosaminyl groups and *A. bisporus* glycans contain sialyl-Galβ and GalNAc groups. Mass spectra of these mushroom species confirm that they have complex *N*- linked glycans.

Türkiye is very rich in mushroom diversity. Unfortunately, there are limited resources in the literature on mushrooms specific to Türkiye. *M. castaneophilus* is a new discovered edible mushroom species in Turkey and its nutritional content has not been investigated yet. In this thesis, protein content and profile of *M. castaneophilus* were shown for the first time. Novel *N*- glycans have been obtained from fruiting body of *M. castaneophilus* mushrooms and characterized by mass spectrometer. In this way, the deficiencies in the literature related to this local mushroom species can be eliminated and the recognition of this mushroom in the scientific community can be increased.

Prebiotics have gained much attention as food ingredients nowadays. Prebiotics are modulating human gut microbioata and host healt. Therefore, researchers are looking for new sustainable alternative new prebiotic sources. Mushrooms are great prebiotic sources because they contain high amount of bioactive polysaccharides, proteins and aminoacids. Nowadays, released *N*- glycans have been considered as potential prebiotic candidates. In this thesis, prebiotic activity of mushroom *N*-glycans have been tested. The growth and development of different Lactobacillus and Bifidobacterium species were investigated in the presence of mushroom *N*-glycans. It has been revealed that *N*- glycans of *A. bisporus*, *P. ostreatus* and *M. castaneophilus* has prebiotic effects on *B. infantis*, *B. kashiwanohonse*, *B. bohemicum*, *L. rhamnosus* and *L. brevis* probiotic bacterias. Also, *A. bisporus* glycans can be used by *L. bulgaricus* as good carbon sources.

In conclusion, mushroom based *N*- glycans have prebiotic effects on some probiotic bacterial species. They are good candidates that can be used in food and pharmaceutical industry. In previous studies, it has been found that glycans obtained from different fungal species can be used in the pharmaceutical industry. The new *N*-glycan structures that are obtained as a result of this thesis can be used in the production of mushroom-based drugs in the future.

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APPENDICES

APPENDIX 1

LADDER THAT USED FOR MUSHROOM SDS-PAGE ANALYSIS