DIPLOMA THESIS

INVESTIGATING THE GROWTH-PROMOTING ACTIVITY OF SPECIFIC POLY- AND OLIGOSACCHARIDES ON LACTOBACILLI.

Maria de los Angeles Ortega Hernández.

Biology, BSc

Supervisors:

Dr. Teréz Barna. Lecturer.

Robert Tupicza. PhD student.

University of Debrecen, Faculty of Science and Technology, Department of Genetics and Applied Microbiology.

Head of the department:

Dr. Ida Gálné Miklós

Debrecen, 2024.

Table of Contents

List of abbreviations

- SEC: Size exclusion chromatography.
- MRS: De Man, Rogosa, Sharpe
- TLC: Thin layer chromatography
- RPM: Revolutions per minute.
- RF: Retention factor
- SEM: Scanning electron micrograph
- MS: Mass spectrometry
- NMR: Nuclear magnetic resonance.

1. Introduction.

1.1. Biotics, gut microbiome and dysbiosis.

Biotics are substances that affect directly the intestine microbiota, including probiotics, prebiotics, symbiotics and postbiotics (Hijová, 2023). They play an important role in improving people's health by influencing the gut microbiome in many beneficial ways. Nowadays the food that is mostly consumed is low in nutrients and high in calories, with a consequence of decreasing the microbiome diversity and leading to a deterioration of health. Therefore, the discovery of new sources of prebiotics and the study of the function of probiotics and microbiome are significant for improving the quality of health of the population.

The **gut microbiome** is made up of trillions of microorganisms, mainly comprising bacteria, from which approximately 95% are anaerobic strains. There are at least 1000 different bacteria species living in the digestive system that are involved in functions critical to our health and well-being. Many factors influence the type and number of bacteria we host and each person has a unique bacterial footprint. (Sankar *et al*., 2015)

The microbiome in the gut is in charge of significant metabolic functions. Essentially, the fermentation and hydrolysis of substances that cannot be digested by the host, synthesis of amino acids, vitamins and short-chain fatty acids and biotransformation of bile acid. Some other beneficial effects attributed to the gut microbiome include enteric nerve regulation, immune-cell and epithelial homeostasis, controlling the proliferation of pathogenic bacteria, brain functions and mood, support of angiogenesis and enteric nerve regulation. Furthermore, through metabolite production and fermentation, it also modulates signalling pathways involved in the homeostasis of the intestinal mucosa. When the balanced interaction between the gastrointestinal tract and the resident microbiota is disrupted, intestinal and extraintestinal gut microbiota diseases may develop. (Vernocchi *et al*., 2016).

Dysbiosis is the term that refers the alteration of the composition of the gut microbiota, in which there is an imbalance of the microorganisms inhabiting the gut of the host. It can be caused by different factors, mainly poor nutrition and dysregulation of the immune system due to psychological and physical stress. It can also be caused by antibiotics, chemotherapy, radiotherapy, hereditary factors and age. Dysbiosis alters the intercellular tight junctions that keep the permeability and integrity of the intestinal mucosa which is essential to prevent the access of pathogens, causing diseases such as allergy, inflammatory bowel disease, obesity, cancer, diabetes, metabolic disorders, cardiovascular dyslipidaemia, and neuropathology. (Tomasello et al., 2016)

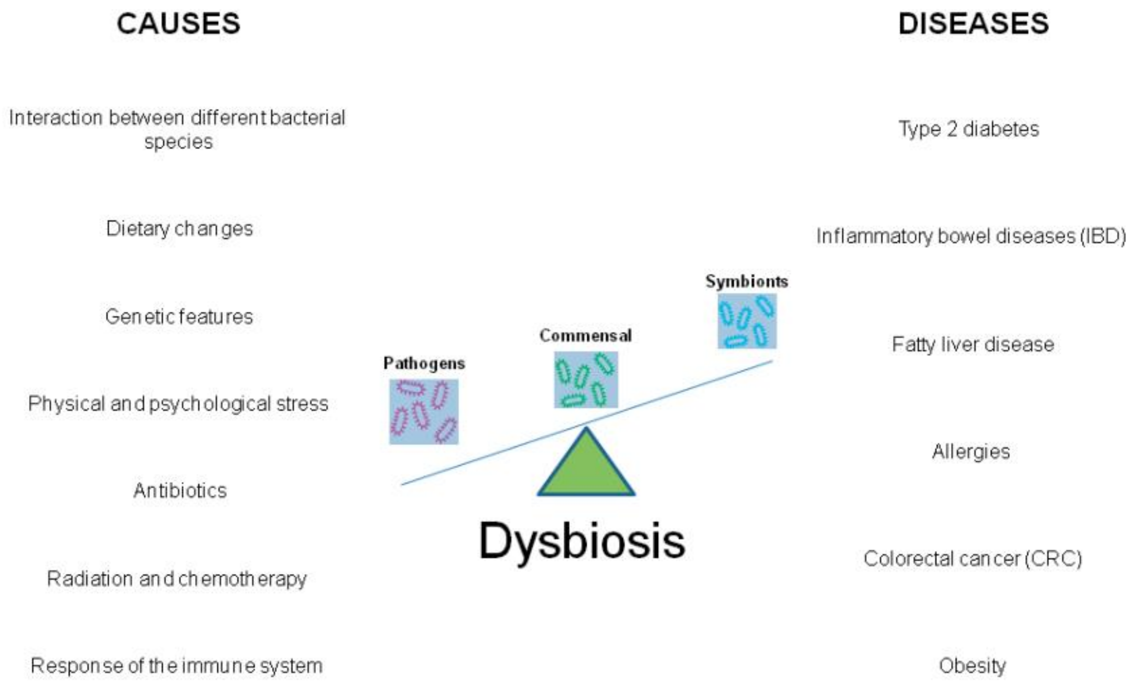


Figure 1: The causes and consequences of dysbiosis. Source: Tomasello *et al*., 2016.

Probiotics are living non-pathogenic microorganisms administrated, that provide modulation and maintenance of intestinal tract health by improving microbial balance. Some of the best-known probiotics belong to the Lactic acid bacteria, a non-homogenous group including several genera of bacteria (*Lactococcus*, ***Lactobacillus***, *Streptococcus,* and *Enterococcus*) and *Bifidobacterium*. They are regulated as dietary supplements and foods (Williams, 2010) . These microorganisms are able to withstand the adverse conditions in the host organism, such as enzymatic action and acidity. They can colonize the host and contribute to health by regulating the microbiome and performing biological functions, like lowering intestinal pH, decreasing colonization and invasion by pathogenic organisms, modifying the host immune response, and synthesizing nutrients. (Silva, 2020)

Prebiotics are nondigestible compounds by the host, but they are metabolized by microorganisms living in the gut, through this they modulate the composition and activity of the gut microbiota giving a beneficial physiologic effect on the host. They form a group of diverse carbohydrates, mostly oligosaccharides that are neither digested nor absorbed in the human small intestine (Quigley, 2019). Prebiotics exhibit health benefits such as reducing the prevalence of intestinal bowel disorders, improving the uptake and bioavailability of minerals, lowering risk factors of cardiovascular diseases, and preventing of obesity, among others. (Pandey *et al*, 2015)

Symbiotics, alluding to the word “synergism” refer to the combined use of prebiotics and probiotics in which the prebiotic compounds favor the probiotic organisms that present health benefits on the host; among them, improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract due to the stimulation of the growth and activation of the metabolism of health-promoting bacteria. More specific health benefits in humans include the improvement of immunomodulating ability, prevention of bacterial translocation, increased levels of probiotic strains and balanced gut microbiota, and improvement of liver function, among others. (Pandey *et al*, 2015)

On the other hand, **postbiotics** are the metabolic products such as polysaccharides, organic acids, peptides, peptidoglycan-derived muropeptides, teichoic acids, organic acids, enzymes, etc. that are secreted by bacteria or released after bacterial lysis. Postbiotics may contribute to the increase of host health by improving specific physiological functions because they are characterized by a clear chemical structure, safety dose parameters, long shelf life, and the content of various signalling molecules that may have antioxidant, immunomodulatory, antihypertensive, anti-inflammatory, and hypocholesterolemic activities, among others. (Aguilar-Toaláa *et al*, 2017)

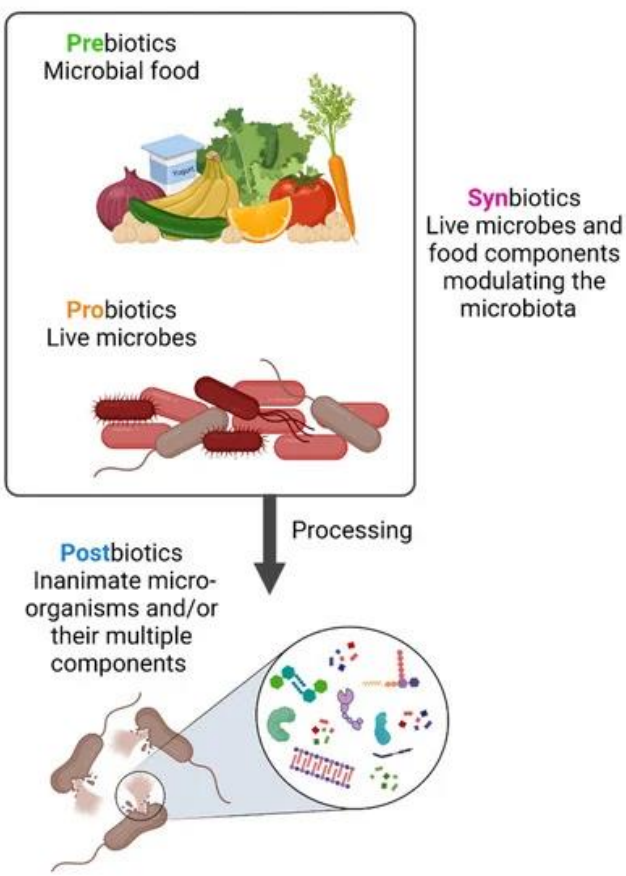


Figure 2: Simple representation of prebiotics, probiotics, symbiotics, and postbiotics. Source: Favero *et al*, 2022.

1.2. *Lentinula edodes* as a potential prebiotic source.

For the research purposes of this Diploma Thesis, *Lentinula edodes* belonging to the *Marasmiaceae* family of fungi, in the order Agaricales and the genus *Lentinula*, was chosen as a source of potential prebiotic preparations. *Lentinula edodes* is the second most popular edible fungus in the world due to its desirable taste and multiple functional activities. Among the components that contribute to the bioactive function of *L. edodes* are β-glucans, specifically Lentinans which have shown antioxidant, antibacterial, immunomodulatory, antitumor, and antiinflammation effects (Dai *et al*, 2023). Lentinan, a β-glucan has two β-(1–6)-glucose branches of five glucose units each on its β-(1–3)-glucose backbone (Figure 3). It is reported to be water-soluble, heat-stable, and alkali-labile in nature. (Chakraborty *et al*, 2023)

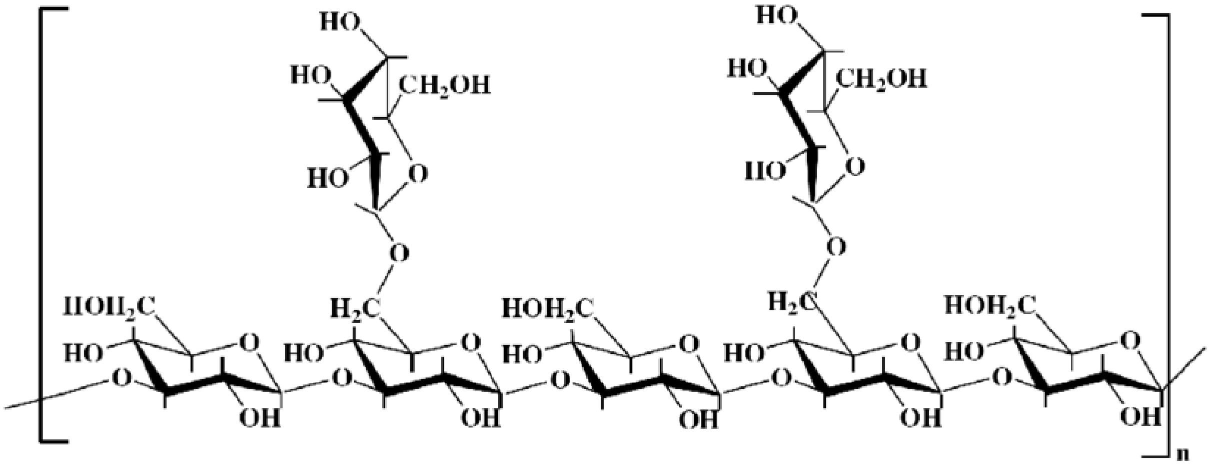
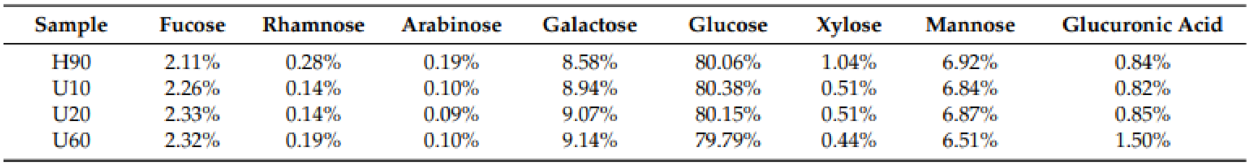


Figure 3: The chemical structure of lentinan. Source: Wang *et al*., 2013.

Recently, a Chinese wild mushroom *Lentinula edodes* was used to isolate *its* β-glucans polysaccharide, which was thoroughly characterized. According to the β-glucans analysis, in addition to the glucose content, the isolated polysaccharide contains several other monosaccharide units as can be seen in Table 1 (Dai *et al*., 2023).

Table 1: The monosaccharide composition (molar ratio, %) of *Lentinula edodes* polysaccharides. The isolation and analysis procedure can be found in the publication of Dai and his co-workers (Dai *et al*., 2023).



Sample	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Xylose	Mannose	Glucuronic Acid
H90	2.11%	0.28%	0.19%	8.58%	80.06%	1.04%	6.92%	0.84%
U10	2.26%	0.14%	0.10%	8.94%	80.38%	0.51%	6.84%	0.82%
U20	2.33%	0.14%	0.09%	9.07%	80.15%	0.51%	6.87%	0.85%
U60	2.32%	0.19%	0.10%	9.14%	79.79%	0.44%	6.51%	1.50%

1.3. *Lactobacillus* species

For the purpose of this study three strains of probiotic bacteria were chosen to test *Lentinula edodes* as a potential prebiotic source, being *Lactobacillus rhamnosus* ATCC 11443, *Lactobacillus plantarum* ATCC 14917, and *Lactobacillus casei* NCAIM B.01526.

As for the most recent modifications in the taxonomy of the mentioned strains, *Lactobacillus casei* has changed to *Lacticaseibacillis casei*, and *Lactobacillus rhamnosus* changed to *Lacticaseibacillus rhamnosus*, which is now recognized as species instead of the previous classification in which it was considered a subspecies of *L. casei*, these two species belong to the

Lacticaseibacillus genus. Moreover, *Lactobacillus plantarum* changed to *Lactiplantibacillus plantarum*, belonging to the *Lactiplantibacillus* genus. These three described species are part of the *Lactobacillaceae* family, the *Lactobacillales* order, and the *Bacilli* class. (Zheng *et al*. 2020). However, for this thesis work, the chosen species are being refered to using the former classification, since those are the names to which the species are refered by the references for this study as well as the company of the purchased bacterial strains.

Lactobacillus casei (Figure 4) and *Lactobacillus rhamnosus* (Figure 5) are species commonly found in the human digestive system, being commercially used to ferment dairy products, producing foods with improved flavor and texture as well as producing bioactive metabolites that confer benefits to the host when consumed. Some of the potentially beneficial mechanisms of these bacteria species include the production of antimicrobial substances such as bacteriocins, enhancing the epithelial barrier through attachment, competition for pathogenic binding sites, and modulation of the immune system. (Hill *et al*, 2018)

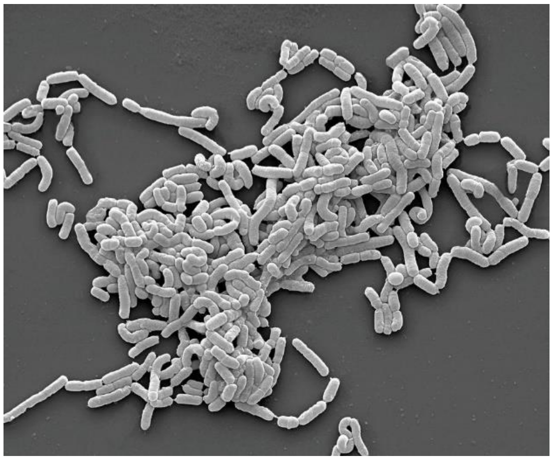


Figure 4: *Lactobacillus casei* bacteria, scanning electron micrograph (SEM). Source: STEVE G. (2018): Science photo Library.

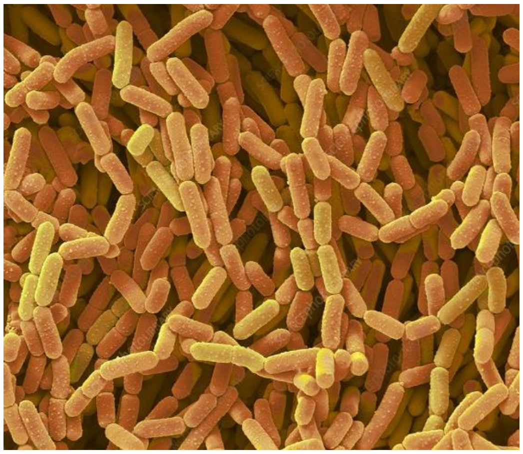


Figure 5: *Lactobacillus rhamnosus* bacteria, coloured scanning electron micrograph (SEM). Source: STEVE G. (2018): Science photo Library.

Lactobacillus plantarum (Figure 6) is a Gram-positive lactic acid bacterium, which is found in fermented food and it is also found in the gastrointestinal tract. It is commonly used in the food industry as a started probiotic, besides this application, *L. plantarum* is widely used in the pharmaceutical industry by contributing significantly to human medicine without any side effects. Such applications can be antioxidant, anticancer, anti-inflammatory, antidiabetic, antiproliferative, and anti-obesity. *L. plantarum* is a non-spore-forming bacterium that produces organic acids such as succinic acid, acetic acid, and lactic acid as major metabolites. This species has antibacterial, antifungal and probiotic properties. (Arasu *et al*, 2015)

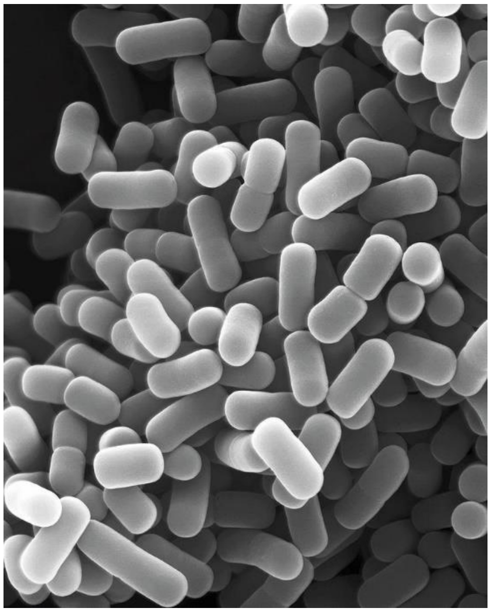


Figure 6: Scanning electron micrograph (SEM) of *Lactobacillus plantarum.* Source: STEVE G. (2018): Science photo Library.

Lactobacillus casei and *Lactobacillus plantarum* strains exhibit probiotic properties such as strong survival in acidic conditions, bile tolerance, cell surface properties, and antagonistic activities for pathogens. (Divyashree *et al*, 2021). *L. casei, L. rhamnosus, and L. plantarum* specifically produce extracellular proteins, bacteriocins, exopolysaccharides, and lipoteichoic acids that influence the physiology and the health of the host by enhancing its immune system, interacting with the epithelial cells and by their antibacterial and antifungal properties. *Lactobacillus* strains are safe for consumption due to their role in the gut defense mechanism and their presence in food. (Arasu *et al*, 2015)

2. Aim of the thesis

This thesis aims to work out an extraction procedure for the carbohydrate content of *Lentinula Edodes* cultivated in Hungary. The further plan is to fractionate the extracted carbohydrate sample according to size, and then test the growth-promoting ability of each fraction on different Lactic acid bacteria such as *Lactobacillus plantarum, Lactobacillus casei,* and *Lactobacillus rhamnosus.*

3. Material and Methods

3.1. Non-Biological Materials

All the chemicals used in this study were analytical grade and purchased from Merck Hungary Kft. Unless otherwise stated.

3.1.1. MRS media

MRS powder from VWR Prolabo Chemicals was used to grow the bacterial strains, composed of 1.0 % Enzymatic digest of casein, 1.0 % meat extract, 0.5% yeast extract, 2.0% glucose, 0.2% dipotassium hydrogen phosphate, 0.5% sodium acetate, 0.2% di ammonium citrate, 0.02% magnesium sulfate, 0.005% manganese sulfate and 0.108% polyoxyethylene-sorbitan.

3.1.1.1. Preparation of Glucose free MRS media

Glucose-free MRS media was prepared by suspending 1.0 % peptone, 1.0 % beef extract, 0.5 % yeast extract, 0.5 % sodium acetate trihydrate, 0.1 % polysorbate 80, 0.2 % dipotassium hydrogen phosphate, 0.2 % triammonium citrate, 0.01 % magnesium sulfate heptahydrate, 0.005 % manganese sulfate tetrahydrate in distilled water. Afterward, the mixture was heated with continuous stirring until the mixture had dissolved, and the pH of the media was checked and adjusted to pH 6.4 ± 0.2 at room temperature. Then, the media was autoclaved at 121°C under pressure for 20 minutes.

3.2. Biological materials

3.2.1. Carbohydrate source

The fruiting bodies of *Lentinula edodes* were purchased from the local market in Debrecen. They were ground as a whole including the caps and stipes then they were freeze-dried. The obtained lyophilized powder was stored at -20 °C.

3.2.2. Bacterial strains

The bacterial strains used for this study were *Lactobacillus rhamnosus* ATCC 11443, *Lactobacillus plantarum* ATCC 14917, and *Lactobacillus casei* NCAIM B.01526. These strains were purchased from the collection of the Hungarian University of Agriculture and Life Sciences and the National Collection of Agricultural and Industrial Microorganisms. The *L. plantarum* strain originally was isolated from pickled cabbage and *L. casei* from pasteurized milk.

3.2.3. Glycoside hydrolase enzymes

Four enzymes (glycoside hydrolases) were used for this study: exo-β-1,4-glucosidase 49290-250MG with genetic origin in *Almond-mandula*, exo-β-1,4-galactosidase G5635-1KU with genetic origin in *Escherichia coli*, endo-β-1,3-glucanase 67138 with genetic origin in *Helix pomatia*, and (cellulase) edo-B-1,4 glucanase 22178 with genetic origin in *Aspergillus niger*. All of them were purchased from the company Sigma-Aldrinch.

3.3. Extraction method of the carbohydrate content from the fruiting bodies of *Lentinula edodes*

3.3.1. Hot water extraction.

Two grams of dried and milled powder of the whole fruiting body were suspended in 100 ml of distilled water and boiled for 4 hours at 100° C under reflux. After that, it was cooled down to room temperature and centrifuged with 4500 rpm for 20 min at room temperature getting Supernatant-I (WSI) and Pellet-I. To precipitate the oligo - and polysaccharides, we added 3 volumes of 97% ethanol to Supernatant-I (WSI); collecting the precipitant Pellet-II and Supernatant-II (WSII). Centrifugation was used on Pellet-II (4500 rpm, at room temperature), whereas the Supernatant-II (WSII) was concentrated by a rotary evaporator, and the dried samples were stored at -20° C. In Figure 4 the chart of the extraction protocol was presented.

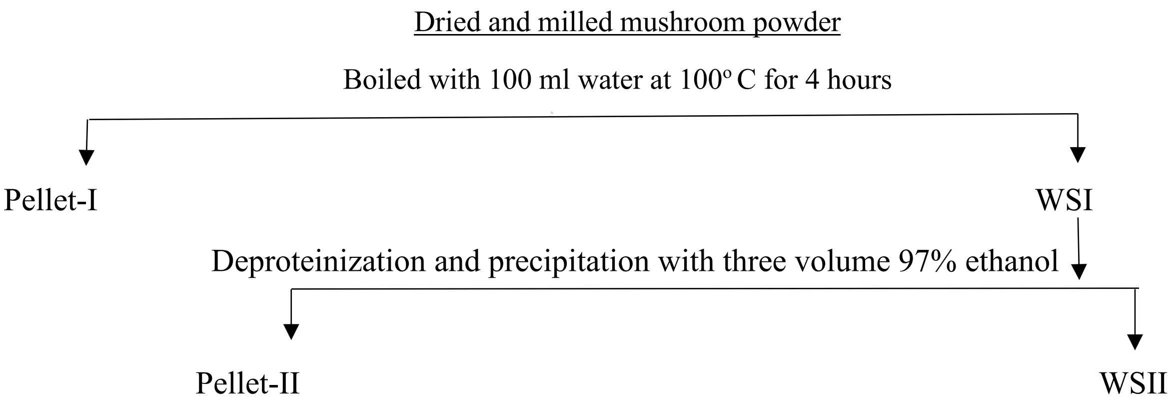


Figure 7: Fractionation of *Lentinula edodes* extracts obtained in hot water extraction procedure.

3.4. Chromatographic separation of substances from the WSII fraction *Lentinula edodes* obtained in hot water extraction

Size exclusion chromatography was applied to separate the *Lentinula edodes* biomolecules from the WSII sample obtained in the hot water extraction protocol. The chromatography was carried out on a chromatographic column (diameter:2.5 cm, length: 82 cm) filled with Bio-Gel P2 resin (Bio-Rad) in the presence of water as an eluant with 0.2 ml/min flow rate. Seven hundred milligrams of the dried Supernatant WSII sample were dissolved in 20 ml distilled water and filtered through a 0.22 µm membrane then uploaded to the column. Eighty-four fractions of 6 ml volume each were collected and analyzed by TLC.

3.5. Analytical techniques used for the separation and characterization of the components in the extracts from *Lentinula edodes*.

3.5.1. Thin layer chromatography.

For the chromatography, TLC silica gel 60 F254 (Merck) was used. First, the TLC was marked with spaces to apply 1 ul sample repeatedly after each drop dried. Then, the plate was developed in an eluting solvent composed of butanol, acetic acid, and water in a 2:1:1 ratio. Afterward, the plate was dried and immersed in a visualization solution for carbohydrates for a couple of seconds, which contained 70 ml of ethanol, 20 ml of distilled water, 3 ml of concentrated sulfuric acid, and 1% orcinol. Finally, the TLC plates were heated up until the bands appeared.

Together with the samples, two carbohydrate standards were added for the identification of the samples. For pentoses, the standard contained xylose, xylobiose, and xylotriose. For hexoses, the standard consisted of mannose, cellobiose, raffinose, maltotetraose, maltopentaose, maltoheptaose.

According to the results of the TLC after the size exclusion chromatography, the next fractions such as 12-19, 20-23, 24-32, 33-41, and 42-45 were combined to give the following fractions, SEC II, SEC, III, SEC IV, SEC, V, and SEC VI, respectively. Then they were stored at -20°C and lyophilized. Those SEC fractions were used for the growth experiment with the *Lactobacillus* strains on microtiter plates. Afterward, another TLC for the analysis of the specific fractions was performed.

3.5.2. Analysis of carbohydrate fractions of *Lentinula edodes* by glycoside hydrolases.

The purified carbohydrate fractions of *Lentinula edodes extract*, namely SEC II, III, IV, V, VI, and Pellet II were hydrolyzed by using four glycoside hydrolases separately on each carbohydrate fraction. The four glycoside hydrolases were: exo-β-1,4-glucosidase, exo-β-1,4-galactosidase, endo-β-1,3-glucanase, and endo-β-1,4-glucanase. The enzymatic reaction volumes for SEC II, V, VI, and Pellet II were 500 µl, whereas for fractions of SEC III and IV were 100 µl. All the reaction mixture contained 5 mg/ml from each of the carbohydrate fractions served as a substrate and 50 mM buffer that was optimal for the specific glycoside hydrolase. In the presence of exo-β-1,4-glucosidase and endo-β-1,4-glucanase enzymes, citrate buffer at pH 5.0 was used. For exo-β-1,4-galactosidase reactions, pH 7.3 was reached by buffering with NaH_2PO_4-Na_2HPO_4. Enzymatic digestions with endo-β-1,3-glucanase were carried out in acetate buffer at pH 5.0. Together with these enzymatic reactions, controls were set up comprising of the carbohydrate substrates without the enzymes.

The mixtures containing exo-β-1,4-glucosidase, exo-β-1,4-galactosidase, and endo-β-1,3-glucanase were incubated at 37°C for 24 hours. Whereas, endo-β-1,4-glucanase catalysed reactions were incubated at 50° C for 24 hours, accordingly. Afterward, TLC analysis was performed to detect the products of the enzymatic hydrolysis.

3.5.3. Estimation of reducing sugar by Somogyi-Nelson method.

Somogyi-Nelson method was used to determine the reducing sugar content of the fungal extract and evaluate the efficiency of the enzyme digestions with all the previously prepared mixtures of mentioned in section 3.7.1.

This method is based on the reduction of Cu^{2+} to Cu^{+} by reducing sugars in an alkaline medium. The resulting Cu^{+} ions react with Arsenomolybdenum hetero-polyacid and are reduced to molybdenum blue. The greater the amount of reducing sugar in the sample, the more intense the blue color.

The required reagents for the experiment are the Copper and the Nelson reagents. For the preparation of the Copper reagent 2 solutions were made, in the first one 12 g of potassium sodium tartrate and 24 g Na_2CO_3 were dissolved in 250 ml H_2O, followed by continuous stirring 4 g $CuSO_4$ x 5 H_2O, and 16 g $NaHCO_3$ were added. For the second one, we dissolved 180 g of Na_2SO_4 in 500 ml hot water and boiled until air bubbles were removed. Then the two solutions were mixed and filtered hot to obtain a clear solution.

To prepare the Nelson reagent two solutions were prepared as well, the first one containing 25 g $(NH_4)_2MoO_4$ x $2H_2O$ in 450 cm^3 H_2O and 21 cm^3 concentrated H_2SO_4. The second one was made by dissolving 3g $AsHNa_2O_4$ x 7 H_2O in 25 cm^3 H_2O. Then both reagents were mixed and incubated for 30 min at 55° C.

To obtain the unknown concentration of the samples from the absorbance, a calibration curve was made with known concentrations of glucose as reducing sugar. For this, first, glucose standards were prepared at concentrations ranging from 0.1 mM to 1.0 mM and a spectral reference containing only water. From the glucose standard, 500 µl was taken to which 500 ul of Copper Reagent was added and the mix was placed in boiling water for 10 minutes. Then 500 ul of Nelson Reagent was added to all the tubes. Consequently, water was added in the same amount of total volume, 1.5 ml, as 2 times dilution. Then, the absorbance was measured at 520 nm and the values were corrected with that of spectral reference. With these data, the calibration curve was constructed.

The same procedures were performed for the fractions extracted from *Lentinula edodes* (SEC II, III, IV, V, VI, and Pellet-II). Based on the obtained absorbance the concentration of the formed reducing sugar was calculated for every fraction using the equation of the calibration curve.

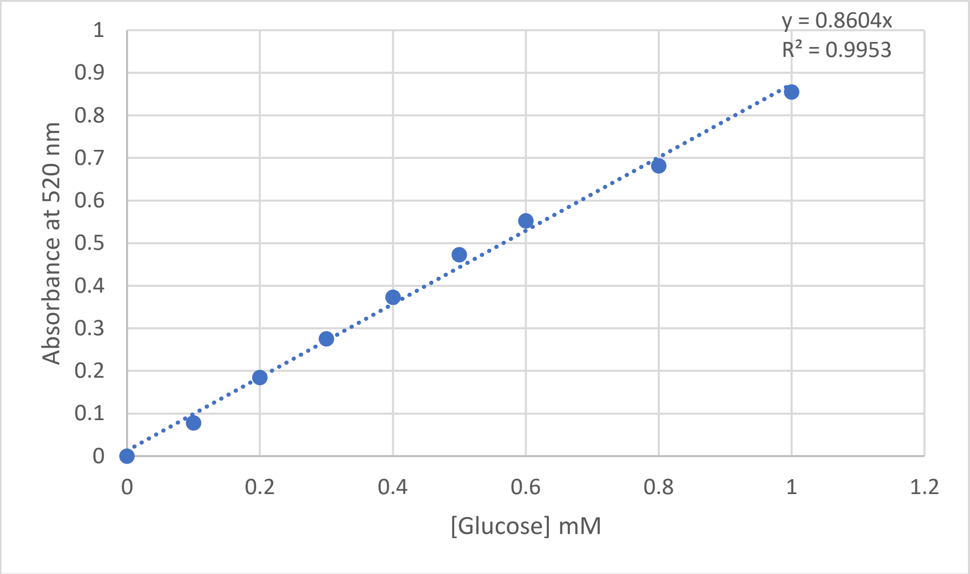


Figure 8: Calibration curve for Somogyi-Nelson reducing reducing sugar assay. Glucose was used as a standard carbohydrate.

3.6. The growth of the *Lactobacillus* bacteria in different environments.

3.6.1. The growth of *L. rhamnosus, L. casei* and *L. plantarum* in MRS media in agar plates

MRS agar media was prepared by dissolving 27.6 g of MRS powder and 7.5 g of agar in (VWR Prolabo Chemicals) 500 ml of distilled water. Then the media was autoclaved at 121° C under pressure for 20 minutes. Approximately 50 ml of MRS agar media was poured into every plate.

The strains from glycerol stock kept at - 80°C were streaked onto MRS agar plates and incubated at 37°C for 48 hours.

3.6.2. Preparation of mini-culture from *Lactobacillus* bacteria

We transferred a single bacterial colony for every species grown on the MRS-agar plate into a test tube containing 5 ml of liquid MRS media each, which was prepared in the same way above mentioned, but without agar. As a control, 5 ml liquid MRS media was used in the absence of bacterial cells. Afterward, they were incubated for 24 hours at 37° C.

3.6.3. Growth of the *Lactobacillus* strains in the presence of the *Lentinula Edodes* fractions as carbon sources in microtiter plates.

All the *Lactobacillus* growth experiments for all the carbohydrate carbon sources were carried out in glucose-free MRS media in triplicates using 96-well microtiter plates. Each valve of the microtiter plate contained 200 µl of glucose-free MRS media, 25 µl of carbohydrate source in which the concentration was 5 mg/ml (50 mg/ml stock solution concentration), 25 µl of *Lactobacillus* bacteria starting culture (where the cell number for *L. rhamnosus* was 15 x 10^4 for *L. casei* 16 x 10^4 and *L. plantarum* 27 x 10^4). Finally, 60 ul of paraffin oil was added at the top.

The tested carbohydrate carbon sources were the fractions II, III, IV, V, and VI, obtained after SEC of the WSII sample from the hot water extraction protocol followed by ethanol precipitation. The growth of three lactic acid bacteria namely, *L. rhamnosus, L. casei,* and *L. plantarum,* was followed in the presence of a specific carbon source mentioned above by measuring the absorbance value at 630 nm in each valve at a constant temperature of 37 °C for 72 hours. Before the absorbance measurement, the microtiter plate was briefly shaken at ~100 rpm.

Along the test measurement, three controls were set up, a valve with glucose-containing MRS media with the bacterial cells used as positive/growth control, carbohydrate-free media supplemented with the bacterial cells applied as negative control, and finally, carbohydrate and bacteria-free MRS medium provided the spectral reference.

4. Results

4.1. Extraction of the carbohydrate content from the fruiting bodies of *Lentinula edodes*.

A hot water extraction procedure of *Lentinula edodes* was worked out consisting of two main steps, four hours boiling period of the finely grained fruiting bodies, then a followed precipitation step using ethanol in a final concentration of 19 v/v % on a water-soluble fraction of the fungal sample (Figure 7). From the initial two-gram dried sample, we obtained final products, 62 mg of Pellet-II and 808 mg of WSII, both of them were solubilized in water getting a final concentration of 50 mg/ml.

4.2. Chromatographic separation of substances from the *Lentinula edodes* extract WSII obtained in hot water extraction

The sample of WSII obtained in the hot water extraction protocol on *Lentinula Edodes* showed the best growth support for all the bacterial stains, therefore this fraction was chosen to be subjected to further chromatographic separation. Size-exclusion chromatography with Bio-Gel P2 resin was applied to purify the WSII sample according to the size of the components. Seven hundred milligrams of the dried WSII samples were separated in eighty-four fractions on a Bio-Gel P2 column. The collected eighty-four fractions were analyzed by TLC. The developed chromatographic plates can be seen in Figure 9.

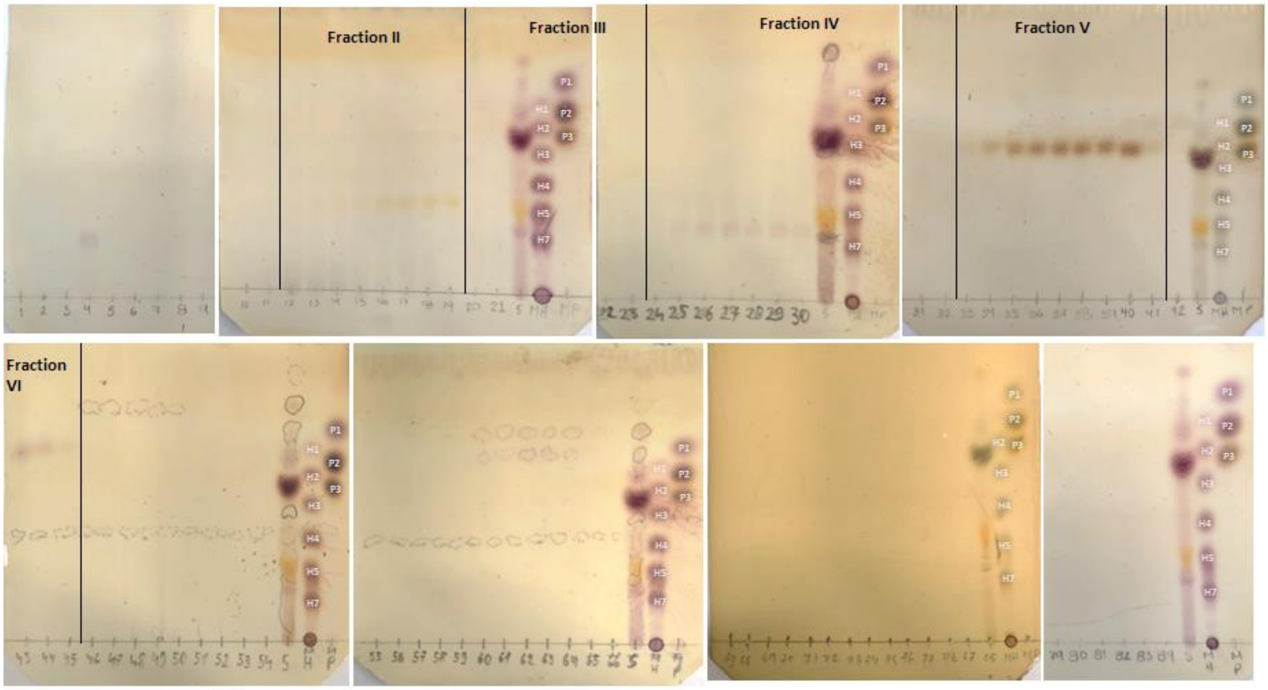


Figure 9: TLC analysis of the SEC fractions from *Lentinula edodes´* WSII sample obtained in the hot water extraction protocol. H- the lane of Hexose standards consisted of mannose (H1), cellobiose (H2), raffinose (H3), maltotetraose (H4), maltopentaose (H5), maltoheptaose (H7). P- the lane of Pentose standards that consisted of xylose (P1), xilobiose (P2) and xilotriose (P3). The point of separation of the fractions according to their composition is signed by the black lines.

According to the TLC analysis (Figure 9), the numbered fractions with identical composition were combined to give five samples named as SEC II, III, IV, V and SEC VI. From these combined fractions another TLC analysis was performed to compare the homogeneity and the size of the obtained five samples (Figure 10). Sec V and Sec VI samples are seemed to be homogeneous and Sec V represents the major component of the extracted WSII fraction from *Lentinula edodes*.

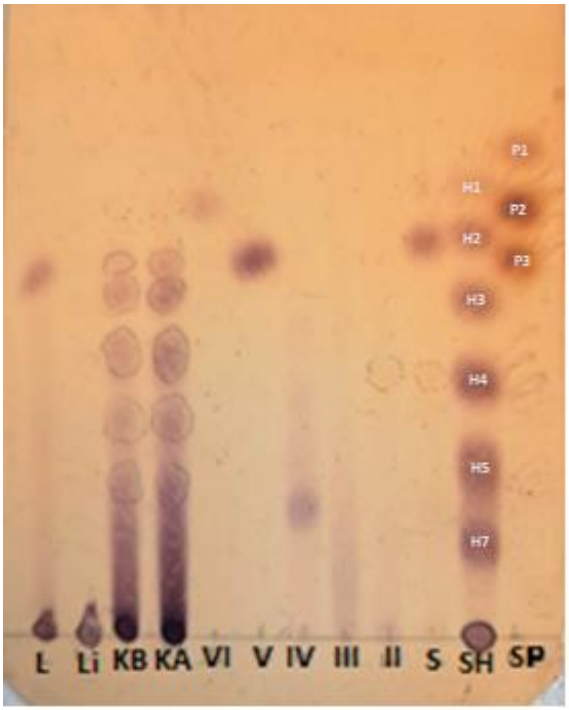


Figure 10: Thin layer chromatography of the combined fractions obtained from the size-exclusion chromatography of *Lentinula edodes* WSII extract and Pellet-II. The Lane II, III, IV, V, VI refer to the samples of SEC II, III, IV, V, and VI. Lane S is WSII (Supernatant-II before chromatography). Lane L is a lentinan standard, Lane Li is Pellet-II; K_B and K_A glucomannan digested by the endo-β-glucanase enzyme. Lane H is a hexose standard consisting of mono- (H1), di- (H2), tri- (H3), tetra- (H4), penta- (H5), and hepta-oligosaccharide (H7) and lane P is a pentose standard comprising of xylose (P1), xilobiose (P2) and xilotriose (P3).

Although the oligomerization degree of unknown oligosaccharides cannot be determined solely on the basis of TLC analysis, it still provides an estimation for applying further separation and spectroscopical techniques in the identification of the molecules. The migration ability of a carbohydrate component depends on the nature of the monosaccharide unit(s) the molecule assembled from and the oligomerization degree, thus comparing the retention factor of our unknown carbohydrate components with that of the applied standard oligosaccharides has a limitation.

SEC-VI contains the smallest carbohydrate components of WSII extract, which run with the highest mobility among the other fractions in between the standard monosaccharide (H1) and the dimer standard (H2) (Figure 10).

The most homogenous fraction SEC-V has an oligosaccharide component band on the TLC plate in between the bands of the dimer (H2) and trimer (H3) oligosaccharide standards.

SEC-IV is not homogenous, although there is a major band with R_f =0.19, which indicates the presence of an oligosaccharide component that might be a hexose because its retention factor is between the R_f value of the pentamer (H5 with R_f =0.26) and the heptamer (H7 with R_f =0.14) oligosaccharide standards.

SEC-III and SEC-II samples are heterogeneous and comprised of larger oligosaccharides. However, the component SEC-II with R_f =0.38 is likely to be a tetraose compared to the R_f of H4 (0.38).

In addition to the chromatographic fractions, WSII, Pellet-II (labeled as Li), which is the other main sample obtained from the alkaline extract protocol of the *Lentinula edodes*, were also analyzed along with lentinan polysaccharide standard (L) on the same TLC plate (Figure 10). Pellet-II (Li) and lentinan (L) standards did not migrate during the condition of the TLC development. They mainly stick to the baseline, and both of the samples can be visualized with the carbohydrate-specific development method. Those results suggest that the Pellet-II is also a polysaccharide as lentinan. Surprisingly, the lentinan standard (L) was not homogenous and oligosaccharide components can be observed in the sample.

4.3. Characterisation of the isolated carbohydrate fractions from *Lentinula edodes* by enzymatic digestion.

A specific enzymatic digestion experiment was carried out on such a polysaccharide that comprised mannose and glucose monomer units, namely glucomannan. The digestion was performed with endo-β-1,4-glucanase enzyme. In Figure 10, lanes K_B and K_A show the result of the enzymatic hydrolysis of glucomannan. Besides the undigested polysaccharide, the liberated oligosaccharides with different lengths were visualized on the TLC plate (Figure 10). Since endo-β-1,4-glucanase specifically cleaves β-1,4-glucosidic bonds, the enzymatic product oligosaccharides are attached through β-1,4-glucosidic bonds within the polysaccharide chain of glucomannan. This experiment indicates that using glycoside hydrolases for the identification of the unknown configuration of the anomeric carbon and the inter-glycosidic linkage in oligo- or polysaccharide is an efficient tool in carbohydrate analysis.

To characterize the inter-glycosidic linkages within the isolated oligo- and polysaccharides from *Lentinula edodes*, four glycoside hydrolases were used in test experiments, two exo-type enzymes: β-1,4-galactosidase, β-1,4-glucosidase and two endo-type enzymes: β-1,3-glucanase, β-1,4-glucanase. The enzymatic digestion experiments were run until the end-point and the hydrolysis was followed by determining the reducing content of the reactions and analyzing the enzymatic products on the TLC plate.

Figure 11 shows the reducing content of the investigated fractions isolated from *Lentinula edodes:* the fractions of the size-exclusion chromatography of WSII samples such as SEC II, III, IV, V, VI and the water-soluble polysaccharide fraction named Pellet-II.

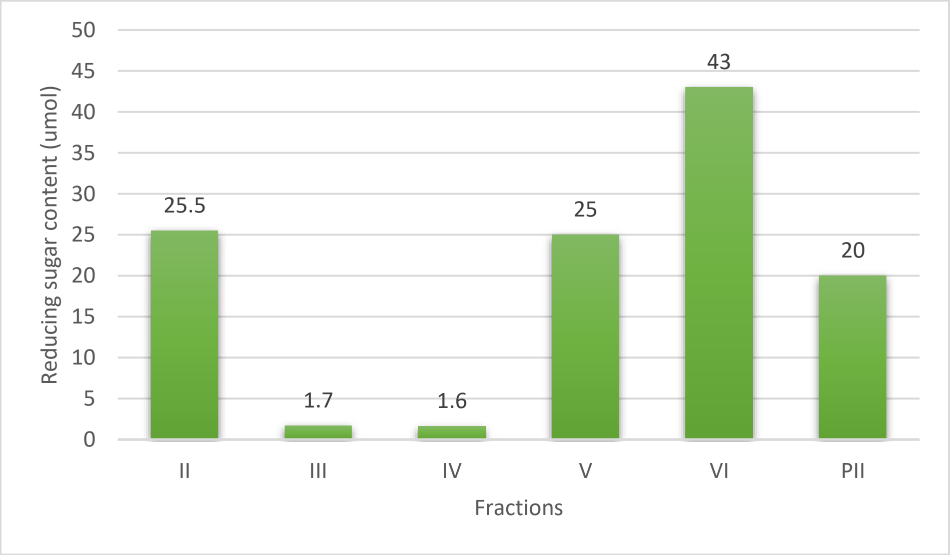


Figure 11: Reducing sugar content of the isolated carbohydrate fractions from *Lentinula edodes* Fractions II, III, IV, V, and VI resulted in the size-exclusion chromatography of WSII sample, PII as Pellet II, the product of the ethanol precipitation from the alkaline extract.

Upon treating the isolated carbohydrate fractions of *Lentinula edodes* individually with the four glycoside hydrolases the increase of the reducing sugar content was determined and expressed in percentage compared to the reducing sugar content measured at the initial time (Figure 12).

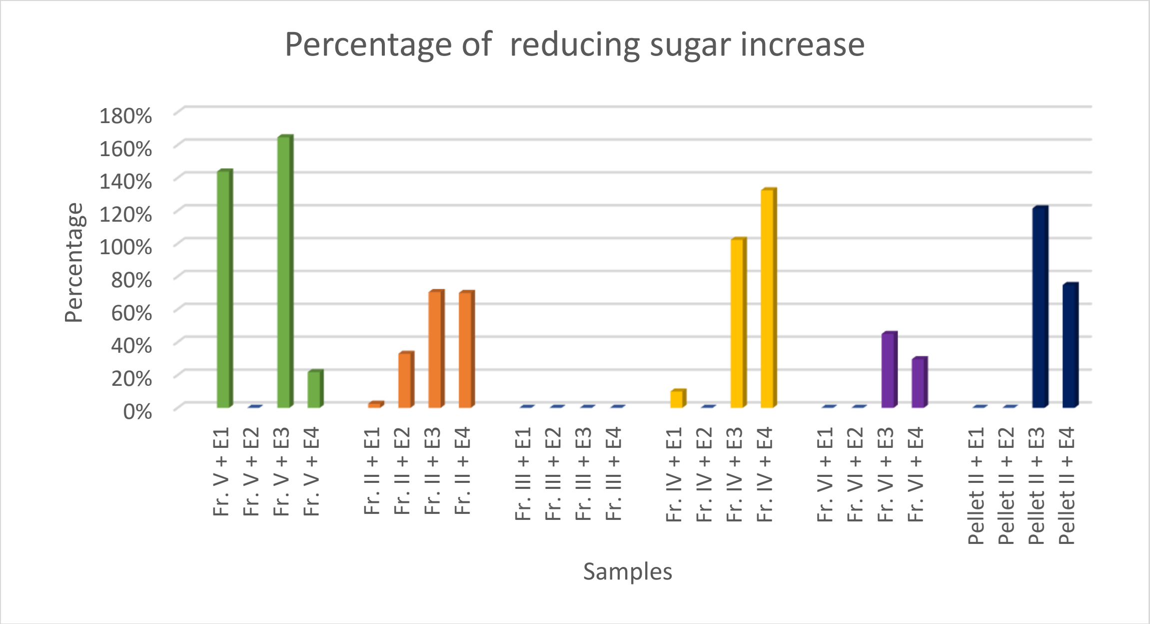


Figure 12: The effect of the glycosidic hydrolases on the carbohydrate fractions of *Lentinula edodes* as an increase in the reducing sugar content in percentage. E1- exo-β-1,4-glucosidase, E2- exo-β-1,4-galactosidase, E3- endo-β-1,3-glucanase, E4- endo-β-1,4-glucanase.

Only in one single case, when the exo-type β-1,4-galactosidase enzyme hydrolyzed the tested fraction, this enzyme was active on SEC II, the inhomogeneous fraction of the WSII sample. SEC II was also hydrolyzed by the two endo-type enzymes. Those results suggest that SEC II contains glucosyl units in β-1,3- and β-1,4- glycosidic linkages as well as β-1,4-galactosyl units. (Figure 12, orange lanes).

The applied four enzymes were not able to digest the SEC III fraction. The two endo-type enzymes hydrolyzed SEC IV and VI, those carbohydrate samples must contain β-1,3- and β-1,4- glucosidic linkages (Figure 12).

On Pellet II both the endo-β-1,3-gluconase (labeled as E3 in Figure 12), and the endo-β-1,4-glucanase (labeled as E4 in Figure 12) show enzymatic activity and produced a high increase of

reducing sugar. Therefore, it can be suggested that the water-soluble polysaccharide fraction, Pellet II, contains β-1,3 and β-1,4 glycosidic bonds.
SEC V was hydrolyzed efficiently with two enzymes including β-1,4-glucosidase and β-1,3-glucanase as well as in a smaller extent with β-1,4-glucanase enzymes.

The TLC analysis has a limitation in the size determination of the carbohydrate components in the investigated carbohydrate fractions isolated from *Lentinula edodes* as can be seen in Figures 9 and 10. Therefore all the isolated samples along with the products that resulted in the enzymatic digestions have been sent for mass spectrometry (MS) analysis. Since the results of the MS analysis have not been obtained in the timescale of the thesis work, an attempt has been made to propose structural components in SEC V samples based on the results of the enzymatic digestion and the TLC analysis.

The component(s) of SEC V migrated in between the applied biose and triose standards on the TLC plate, in the described development conditions, as can be seen in Figure 10. Assuming that the components in SEC V are trisaccharide units and knowing that the exo-type β-1,4-glucosidase, which cuts glycosidic bond from the non-reducing end, and the endo-type β-1,3-glucanase hydrolyzed the sample, the proposed structure is β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-D-glucopyranose (trivial name: cellobiosyl-(1→3)-β-D-glucose), that can be seen in Figure 13.

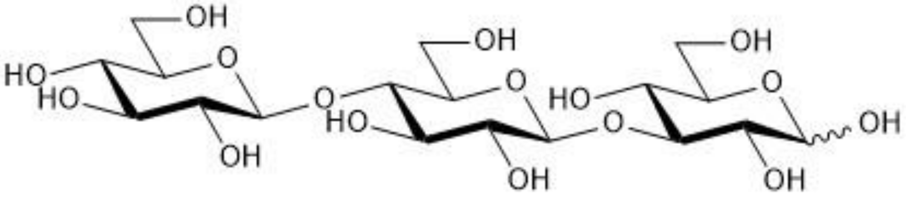


Figure 13: The structure of the proposed trisaccharide present in SEC V: cellobiosyl-(1→3)-β-D-glucose

If we assume that SEC V constitutes disaccharides, the results of the enzymatic digestions on SEC V can be interpreted as the existence of two different biose molecules such as cellobiose, where the two glucopyranosyl moieties are linked *via* 1,4- β-D-glycosidic bond and laminarabiose, β-D-glucopyranosyl-(1→3)-D-glucopyranose (trivial name: laminarabiose) (Figure 14)

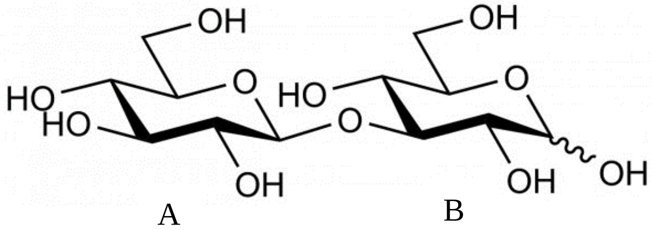


Figure 14: The structure of the proposed disaccharides present in SEC V: cellobiose (A) and laminarabiose (B)

The isolated polysaccharide fraction Pellet II was hydrolyzed by the two endo-type enzymes, which advocates that this polysaccharide constitutes β-1,4- and β-1,3 glycosidic linkages (Figure 12).

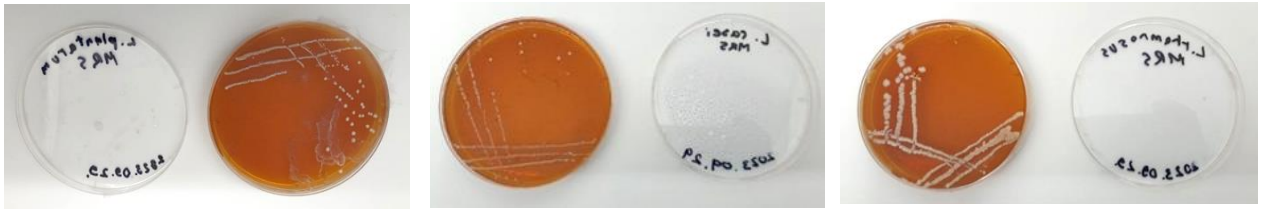
In order to identify the isolated carbohydrate components of the *Lentinula edodes* further investigations are required such as applying further separation techniques to gain homogenous entities followed by using complex techniques in NMR spectroscopy to reveal the molecular structures. This is out of the scope of this work.

4.4. Testing the growth of the *Lactobacillus* bacteria in the presence of the isolated carbohydrate fractions from *Lentinula edodes* in microtiter plate experiments.

4.4.1. The growth of *Lactobacillus* bacteria in the presence of oxygen

The three *Lactobacillus* species used in this study are oxygen-tolerant anaerobes with fermentative metabolism (Zotta et. al. 2017). Both on agar-MRS and liquid MRS media, the three investigated bacteria, *Lactobacillus rhamnosus* ATCC 11443, *Lactobacillus plantarum* ATCC 14917, and

Lactobacillus casei NCAIM B.01526, were grown successfully in the presence of oxygen as can be seen in Figures 15 and 16.



1. *L. plantarum* 2. *L. casei* 3. *L. rhamnosus*

Figure 15: MRS Agar plates with bacterial colonies.

Starter cultures for microtiter plate experiments were also made successfully by transferring a single colony from each agar plate into liquid MRS media in the presence of oxygen (Figure 16).

Figure 16: Starter cultures of the three *Lactobacillus* species grown in MRS media, in the presence of oxygen (the three tubes on the left) and a control media in the absence of bacterial cells.

4.5. Following the bacterial growth in the presence of different carbon sources in microtiter plate experiments.

The isolated carbohydrate fractions from *Lentinula edodes* were investigated as carbon sources to test their growth support on the three *Lactobacillus* species such as *L. rhamnosus* ATCC 11443, *L. plantarum* ATCC 14917 and *L. casei* NCAIM B.01526. The bacterial growth test experiments were carried out in glucose-free MRS medium, in microtiter plates, at constant temperature measuring OD_{630} in time intervals. For the estimation of the growth support, glucose-added MRS media was used as a positive control, and glucose- and other carbohydrate-free MRS media was used as a negative control. For every growth curve, the procedure was carried out in triplicate, from which the mean was calculated and the cell numbers were plotted as the function of time.

The growth of *L. casei* was supported significantly by SEC V and VI fractions even in the comparison with glucose. The plateau of the stationary phases for the two growth curves of SEC V and VI have reached the same value, however, the slope of the exponential phase for the SEC V growth curve is slightly higher than that of SEC VI (Figure 17). The lag phases for the positive control and the SEC V and VI growth curves are similar indicating that the cellular components were already present to metabolize these carbohydrate components by the bacteria. We can also observe that WSII supports the growth of the bacteria, but in a lower degree than the SEC fractions V and VI. Therefore, the main metabolized components of WSII are found in SEC V and VI (Figure 17).

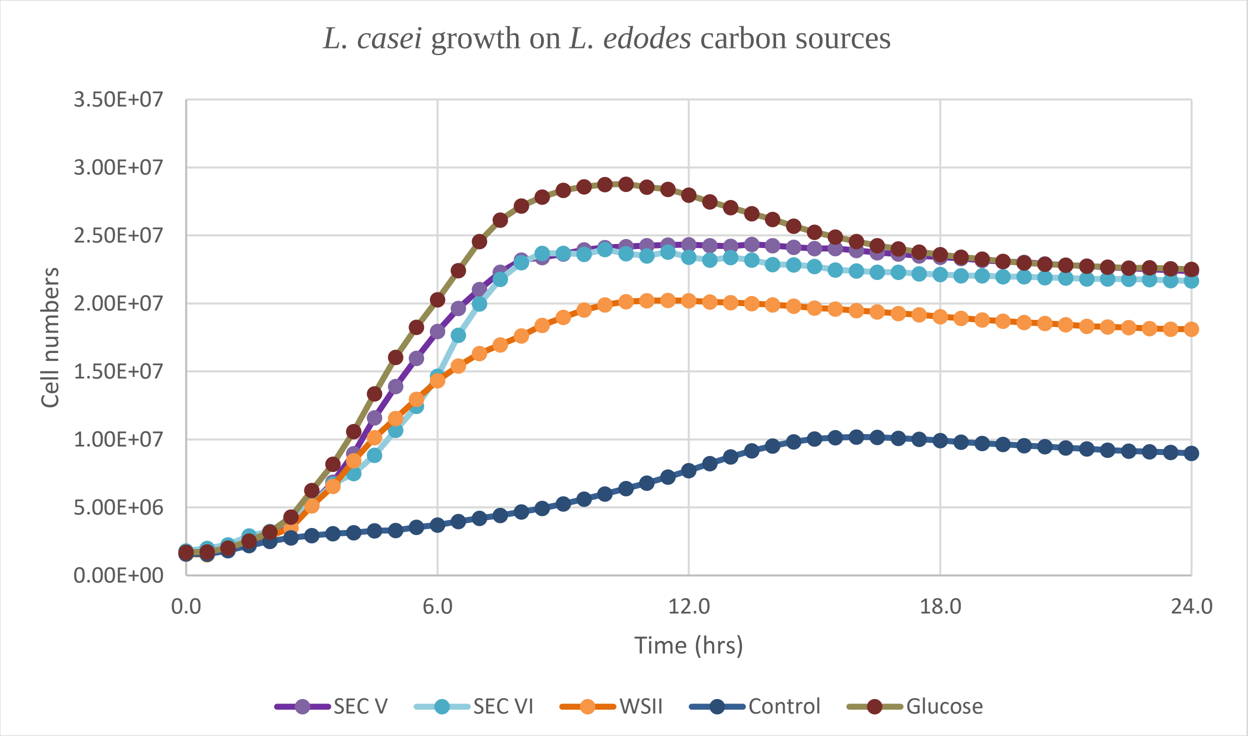


Figure 17: The growth curves of *L. casei* measured in the presence of the isolated fractions SEC V, SEC VI, and WSII from *L. edodes*
Control (Blue): *L. casei* was grown on carbohydrate-free MRS medium.

SEC fractions II, III, and IV did not support the growth of *L. casei*, since the observed growth curves seemed to be identical to the growth curve produced by the negative control (Figure 18).

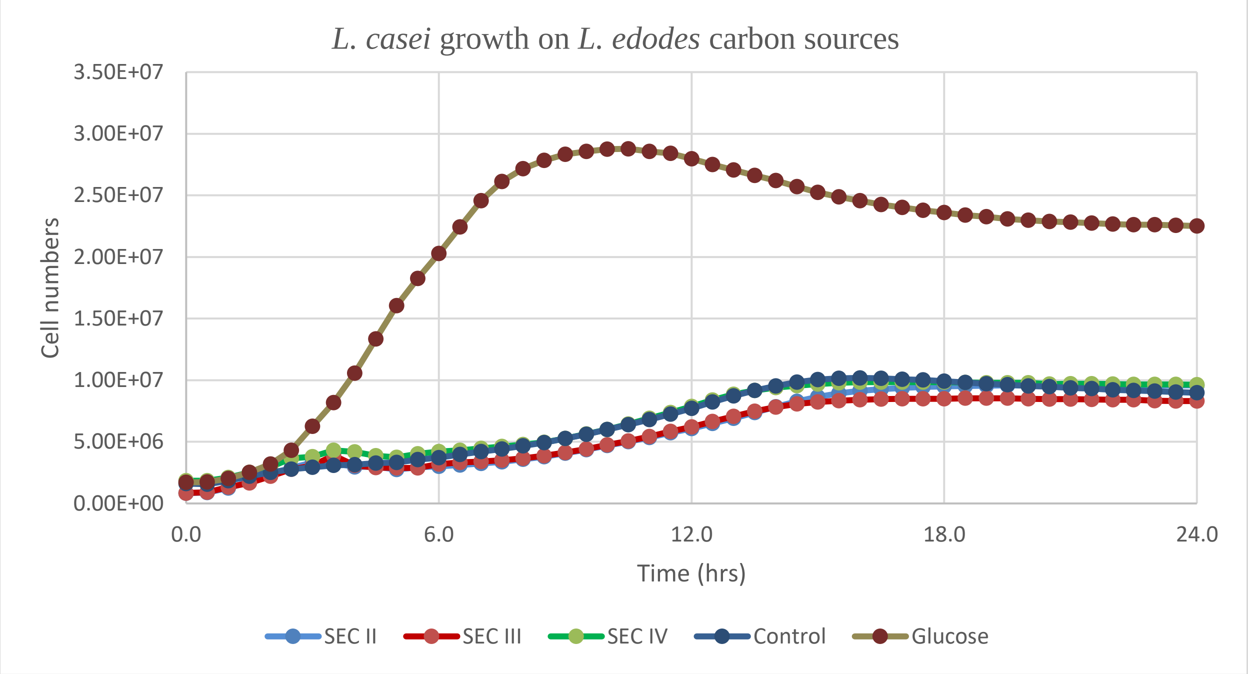


Figure 18: Growth curves of *L. casei* measured in the presence of the isolated fractions II, III and IV from *L. edodes*.
Control (Blue): *L. casei* was grown on a carbohydrate-free MRS medium.

For *L. rhamnosus*, glucose provided the far best growth support, followed by the SEC fractions VI and V (Figure 19).

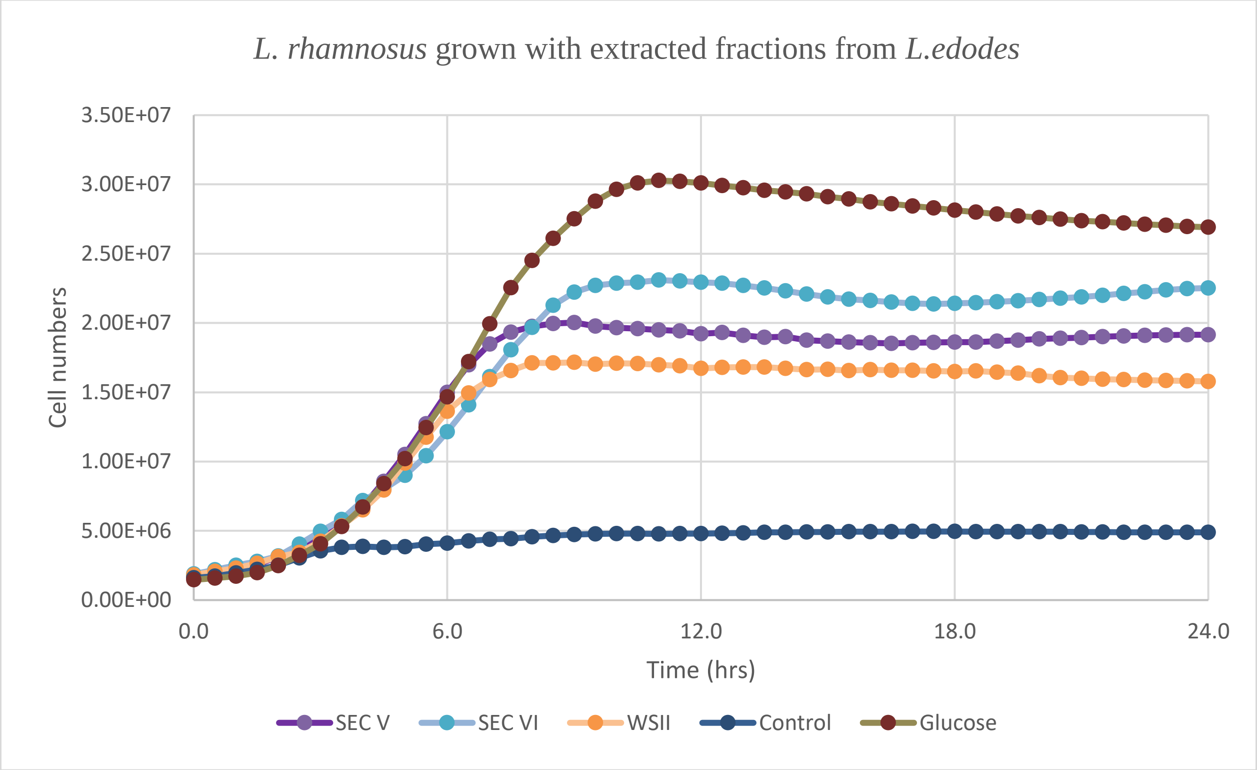


Figure 19: Growth curves of *L. rhamnosus* measured in the presence of SEC fractions V and VI extracted from *L. edodes*
Control (Blue): *L. rhamnosus* was grown on carbohydrate-free MRS medium

SEC II, III, and IV fractions were not metabolized by *L. rhamnosus* producing similar growth curves to that of the negative control (Figure 20). Based on these results, we can conclude that the carbohydrate-active components of *L. rhamnosus* are SEC VI and V present in WSII.

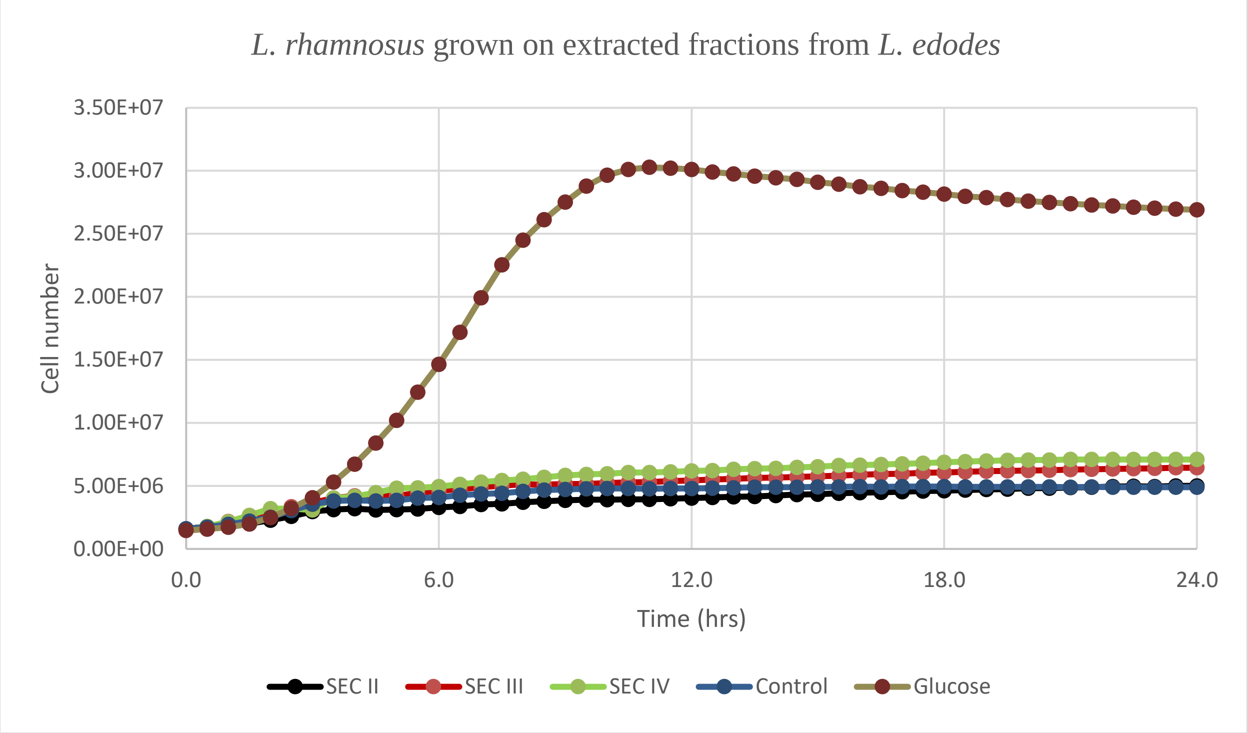


Figure 20: Growth curves of *L. rhamnosus* measured in the presence of SEC II, III and IV fractions isolated from *L. edodes*.
Control (Blue): *L. rhamnosus* was grown on carbohydrate-free MRS medium

For *L. plantarum*, SEC V provided the best growth support, which was followed by SEC VI and glucose (Figure 21). The latter two reached a very similar plateau (Figure 21).

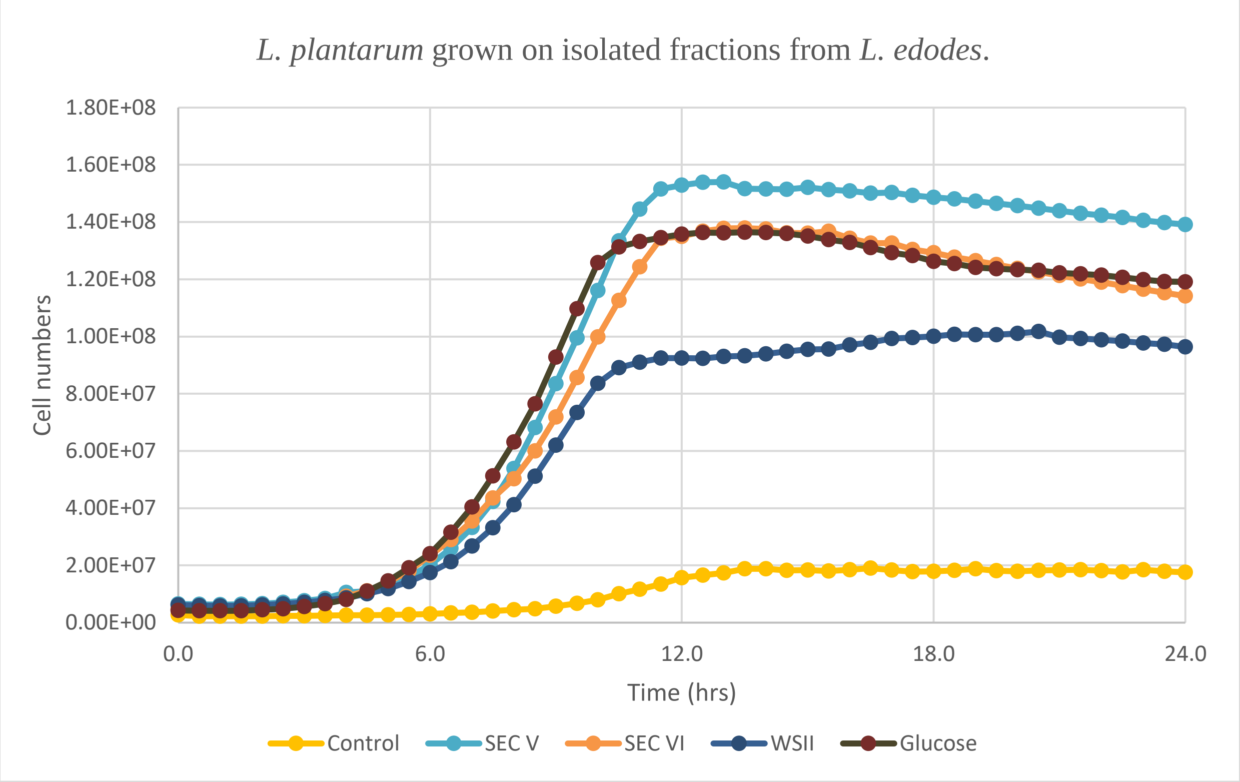


Figure 21: Growth curves of *L. plantarum* measured in the presence of SEC fractions V and VI extracted from *L. edodes*.
Control (Blue): *L. plantarum* was grown on carbohydrate-free MRS medium

In Figure 22 we can observe that *L. plantarum* was able to grow slightly in the presence of SEC IV, while SEC fractions II and III show a similar plateau to that of the control grown in carbohydrate-free media, indicating that those fractions do not support the growth of *L. plantarum*.

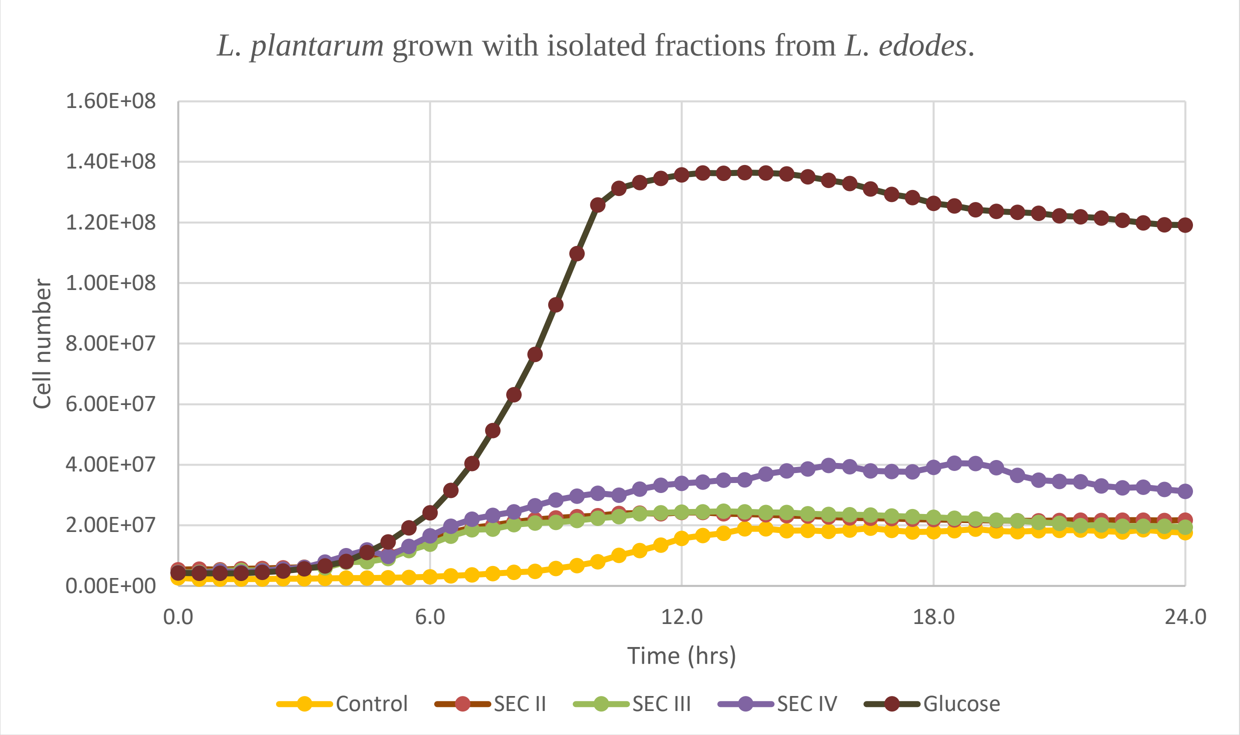


Figure 22: Growth curves of *L. plantarum* measured in the presence of SEC II, III and IV fractions isolated from *L. edodes*
Control (Blue): *L. plantarum* was grown on carbohydrate-free MRS medium

Pellet II, the water-soluble polysaccharide fraction did not support the growth of any of the investigated *Lactobacillus* strains.

5. Discussion

In this thesis, one of the aims was to isolate the carbohydrate content of the edible mushroom, from *Lentinula edodes* harvested in Hungary. For this task, an extraction protocol was developed, which consisted of two main steps, a four-hour-long hot water extraction step, which was followed by an ethanol precipitation treatment of the water-soluble fraction. In this procedure, two main fractions were obtained (named WSII and Pellet-II): the one that contains mainly oligosaccharide components and the other main fraction, which is a water-soluble polysaccharide as it was proved by TLC analysis. The sample (WSII) containing the mixture of oligosaccharides was separated by size-exclusion chromatography to fractionate the oligosaccharide components according to their size. This technique resulted in six fractions, which were further characterized and subjected to enzymatic digestion by four glycoside hydrolases. Among the glycoside hydrolases, there were two exo-acting and two endo-acting enzymes. The enzyme activities on the six carbohydrate fractions from *Lentinula edodes* were followed by reducing sugar determination during the enzymatic reactions. Since the enzymes cleave specific interglycosidic linkage, we were able to propose the presence of monosaccharide units with specific anomeric carbon configuration in the oligosaccharide components of the different chromatographic fractions (names as SEC-II, - III, -IV, -V and -VI) and in the isolated polysaccharide (Pellet-II).

The second aim of the thesis was to test the carbohydrates isolated from *Lentinula edodes*, in case of applying a successful extraction method, whether they were able to promote the growth of *Lactobacillus* species. Protocols were worked out for following the growth of three bacteria, *L. plantarum*, *L. casei* and *L. rhamnosus* in microtiter plate experiments. The six oligosaccharide fractions were applied as carbon sources for the *Lactobacillus* bacteria, there were two carbohydrate fractions (SEC-V and SEC-VI) metabolized by *L. plantarum*, *L. casei* and *L. rhamnosus* as well. After determination and comparison of the growth curve for each of the growth experiments when different *Lactobacillus* species were grown on different carbohydrate sources, the oligosaccharide(s) present in SEC-V showed the most promising *Lactobacillus*-growth promoting ability on all species. The water-soluble polysaccharide fraction cannot be metabolized by the examined *Lactobacillus* species.

In future work, thorough molecular characterization using separation techniques followed by spectroscopical techniques such as NMR spectroscopy is required for the identification of the different carbohydrate molecules, which supported the growth of the tested bacteria.

In the literature, the majority of the publications described and characterized β-glucan polysaccharides from *Lentinula edodes*, which derived from a water-insoluble fraction and proved several beneficial biological activities (Chakraborty *et al*, 2023). Very few publications dealt with the oligosaccharide contents of *Lentinula edodes* and their impact on different biological systems. Therefore, the subject of the thesis is worth further investigating on a deeper scale to reveal the unknown significance of potential prebiotics.

6. Acknowledgments

First of all, I would like to express my sincere gratitude towards the supervisor of this thesis work, Dr. Teréz Barna from whom I have learned the most during my bachelor's degree. She provided guidance and support at every stage of this work. Secondly, I thank the PhD student Róbert Tupicza for all his help, patience, and kindness during the laboratory work and guidance when writing the thesis.

Lastly, I would like to thank all the people who supported me and believed in me during the thesis work.

7. Bibliography

AGUILAR-TOALÁ, J. E., GARCÍA-VARELA, R., GARCÍA, H. S., MATA-HARO, V., GONZÁLEZ-CÓRDOVA, A. F., VALLEJO-CORDOBA, B., & HERNÁNDEZ-MENDOZA, A. (2018): Postbiotics: An evolving term within the functional foods field. Trends in Food Science & Technology. 75. 105–114. https://doi.org/10.1016/j.tifs.2018.03.009

ARASU, M. V., AL-DHABI, N. A., ILAVENIL, S., CHOI, K. C., & SRIGOPALRAM, S. (2016): In vitro importance of probiotic Lactobacillus plantarum related to medical field. Saudi journal of biological sciences. 23. 1. S6–S10. https://doi.org/10.1016/j.sjbs.2015.09.022

CHAKRABORTY, S., BEURA, M., SHARMA, S. K., SINGH, A., DAHUJA, A., & KRISHNAN, V. (2023): Lentinan, β-glucan from Shiitake (Lentinula edodes): A review on structure, conformational transition, and gastro-intestinal interaction contributing towards its anti-diabetic potential. Trends in Food Science & Technology. 142, 104224. https://doi.org/10.1016/j.tifs.2023.104224

DAI, Y., WANG, L., CHEN, X., SONG, A., HE, L., WANG, L., & HUANG, D. (2023): Lentinula edodes Sing Polysaccharide: Extraction, Characterization, Bioactivities, and Emulsifying Applications. Foods (Basel, Switzerland). 12. 17. 3289. https://doi.org/10.3390/foods12173289

DENNIS K. (2018): Science photo Library. Scanning electron micrograph (SEM) of *Lactobacillus plantarum.* https://sciencephotogallery.com/featured/1-lactobacillus-plantarum-dennis-kunkel-microscopyscience-photo-library.html Downloaded: 19/04/2017

DIVYASHREE, S., ANJALI, P., SOMASHEKARAIAH, R., & SREENIVASA, M. Y. (2021): Probiotic properties of Lactobacillus casei – MYSRD 108 and Lactobacillus plantarum-MYSRD 71 with potential antimicrobial activity against Salmonella paratyphi. Biotechnology Reports. 32. e00672. https://doi.org/10.1016/j.btre.2021.e00672

FAVERO, C., GIORDANO, L., MIHAILA, S. M., MASEREEUW, R., ORTIZ, A., & SANCHEZ-NIÑO, M. D. (2022): Postbiotics and Kidney Disease. Toxins. 14. 9. 623. https://doi.org/10.3390/toxins14090623

HIJOVÁ E. (2023): Benefits of Biotics for Cardiovascular Diseases. International journal of molecular sciences. 24. 7. 6292. https://doi.org/10.3390/ijms24076292

HILL, D., SUGRUE, I., TOBIN, C., HILL, C., STANTON, C., & ROSS, R. P. (2018): The Lactobacillus casei Group: History and Health Related Applications. Frontiers in microbiology. 9. 2107. https://doi.org/10.3389/fmicb.2018.02107

PANDEY, K. R., NAIK, S. R., & VAKIL, B. V. (2015): Probiotics, prebiotics and synbiotics- a review. Journal of food science and technology. 52. 12. 7577–7587. https://doi.org/10.1007/s13197-015-1921-1

QUIGLEY E. M. M. (2019): Prebiotics and Probiotics in Digestive Health. Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association. 17. 2. 333–344. https://doi.org/10.1016/j.cgh.2018.09.028

SANKAR, S. A., LAGIER, J. C., PONTAROTTI, P., RAOULT, D., & FOURNIER, P. E. (2015): The human gut microbiome, a taxonomic conundrum. Systematic and applied microbiology. 38. 4. 276–286. https://doi.org/10.1016/j.syapm.2015.03.004

SILVA, D. R., DE CÁSSIA ORLANDI SARDI, J., DE SOUZA PITANGUI, N., ROQUE, S. M., DA SILVA, A. C. B., & ROSALEN, P. L. (2020): Probiotics as an alternative antimicrobial therapy: Current reality and future directions. Journal of Functional Foods. 73. 104080. https://doi.org/10.1016/j.jff.2020.104080

STEVE G. (2018): Science photo Library. *Lactobacillus casei* bacteria, scanning electron micrograph (SEM). https://sciencephotogallery.com/featured/lactobacillus-casei-bacteria-steve-gschmeissnerscience-photo-library.html Downloaded: 19/04/2017

STEVE G. (2018): Science photo Library. *Lactobacillus rhamnosus* bacteria, coloured scanning electron micrograph (SEM). https://sciencephotogallery.com/featured/1-lactobacillus-rhamnosus-bacteria-steve-gschmeissnerscience-photo-library.html Downloaded: 19/04/2017

TOMASELLO, G., MAZZOLA, M., LEONE, A., SINAGRA, E., ZUMMO, G., FARINA, F., DAMIANI, P., CAPPELLO, F., GERGES GEAGEA, A., JURJUS, A., BOU ASSI, T., MESSINA, M., & CARINI, F. (2016): Nutrition, oxidative stress and intestinal dysbiosis:

Influence of diet on gut microbiota in inflammatory bowel diseases. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia. 160. 4. 461–466. https://doi.org/10.5507/bp.2016.052

VERNOCCHI, P., DEL CHIERICO, F., & PUTIGNANI, L. (2016): Gut Microbiota Profiling: Metabolomics Based Approach to Unravel Compounds Affecting Human Health. Frontiers in microbiology. 7. 1144. https://doi.org/10.3389/fmicb.2016.01144

WANG, H., LIU, Y. M., QI, Z. M., WANG, S. Y., LIU, S. X., LI, X., WANG, H. J., & XIA, X. C. (2013): An overview on natural polysaccharides with antioxidant properties. Current medicinal chemistry. 20. 23. 2899–2913. https://doi.org/10.2174/0929867311320230006

WILLIAMS N. T. (2010): Probiotics. American journal of health-system pharmacy: AJHP: official journal of the American Society of Health-System Pharmacists. 67. 6. 449–458. https://doi.org/10.2146/ajhp090168

ZHENG, J., WITTOUCK, S., SALVETTI, E., FRANZ, C. M. A. P., HARRIS, H. M. B., MATTARELLI, P., O'TOOLE, P. W., POT, B., VANDAMME, P., WALTER, J., WATANABE, K., WUYTS, S., FELIS, G. E., GÄNZLE, M. G., & LEBEER, S. (2020): A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. International journal of systematic and evolutionary microbiology. 70. 4. 2782–2858. https://doi.org/10.1099/ijsem.0.004107

ZOTTA, T., PARENTE, E., & RICCIARDI, A. (2017): Aerobic metabolism in the genus Lactobacillus: impact on stress response and potential applications in the food industry. Journal of applied microbiology. 122. 4. 857–869. https://doi.org/10.1111/jam.13399