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Research Article

Investigating the effect of prebiotic polysaccharides from algae and mushrooms on probiotic activity

Amirreza Rezaei¹, Mohaddeseh Larypoor^{*2}, Mozhgan Emtiazjoo³

1- Master of Microbial Biotechnology, Department of Biotechnology, NT.C., Islamic Azad University, Tehran North Branch, Tehran, Iran

2- Department of Microbiology, NT.C., Islamic Azad University, Tehran North Branch, Tehran, Iran

3- Department of Marin Biology, NT.C., Islamic Azad University, Tehran North Branch, Tehran, Iran

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ABSTRACT

For human food safety, fungal or algal sources are used instead of plant sources. The aim of this research is to investigate the effects of prebiotic polysaccharides from *Ganoderma*, *Lentinula*, various fungi, *Spirulina*, *Chlorella*, and other algae on probiotics isolated from water and soil samples. Samples were collected from different traditional dairies. Microscopic, macroscopic, and biochemical tests, along with molecular methods, were used to identify bacterial and yeast isolates. Isolated strains were evaluated for their resistance to heat, acid, bile salts, and antibiograms to assess their probiotic potential. Polysaccharides were extracted from algae and fungi using the phenol-sulfuric acid method. The amount of polysaccharides extracted was then measured, and their structures were identified using FT-IR analysis. Subsequently, the effects of polysaccharides on the growth and postbiotic production of probiotic isolates were investigated to assess potential synergistic effects. Technical abbreviations will be explained when first introduced. Four strains—*Pichia* sp. strain HBUM07161, *Saccharomyces cerevisiae* strain CEN.PK113-7D, *Lactobacillus gallinarum* strain ATCC 33199, and *Loigolactobacillus coryniformis* were identified during laboratory and molecular tests for their probiotic properties. The polysaccharides extracted from *Ganoderma*, *Lentinula*, *Spirulina*, and *Chlorella* algae had a stronger impact on growth rate and organic acid production as postbiotics compared to inulin, a standard prebiotic. The incorporation of biopolymers derived from algae and moss as prebiotics can inhibit the reduction of plant-based food sources. Moreover, due to their biocompatibility and non-toxicity, they provide suitable growth substrates for probiotics such as *Ganoderma lucidum*, *Lentinula edodes*, *Spirulina*, and *Chlorella*.

1. Introduction

Considering the widespread production and supply of synbiotic foods, it is very necessary to study and investigate different sources of extraction of nutraceuticals for the production of these products. Various researches have shown the positive effect of these supplements on human health and various prevention and treatment. Research into the production and

supply of commercial supplements such as inulin started years ago and now have a significant share of the synbiotic food market. However, new supplements such as polysaccharides of bacterial origin, yeasts, edible fungi and all types of algae are a new area of research in synbiotic supplements due to their economic efficiency and numerous proven

*Corresponding author: Mohaddeseh Larypoor
E-mail address: m.larypoor@iau.ir

benefits for human health (Ataollahi et al., 2021). Currently, extensive research has been devoted to foods that contain probiotic strains. Probiotics are known as live microorganisms, and when consumed in appropriate amounts, they have a beneficial effect on the health of the host. Despite the fact that lactic acid bacteria and *Bifidobacteria* are mainly probiotic microorganisms, several yeasts such as *Saccharomyces* and *Kluyveromyces* strains are also used for their properties as probiotics. Due to being resistant to antibiotics, yeasts also show the ability to exchange with probiotic bacteria (Abolghasemi et al., 2022). Prebiotics are defined by Gibson and Roberfreud in 1995 as indigestible or poorly digestible compounds that multiply against digestive enzymes in the human colon. According to this definition, a prebiotic has been selected as a fermented substance that has beneficial effects on the health of the host by making specific changes to the composition or activity of the microbiota of the digestive system (Saad et al., 2013). Edible-medicinal fungi are mainly used in Asian countries to prevent various diseases. These fungi are also used to treat lung diseases and cancer. *Ganoderma lucidum* and *Lentinula edodes* are among the most important edible-medicinal fungi.

The polysaccharides of these fungi are one of the bioactive compounds with anti-cancer properties (Larypoor et al., 2021). Algae have been used for food and medicine for more than two thousand years. *Spirulina* blue-green algae is multicellular and filamentous with rapid growth. It is able to grow at a temperature above 20 degrees Celsius in salty and alkaline waters with the presence of carbonate, bicarbonate and inorganic nitrogen (Andrade et al., 2007). Another alga used in this research is *Chlorella*, one of the most famous microalgae. These microscopic algae, with a diameter of 2 to 10 microns, live in fresh water. Similar to plants, *Chlorella* is one of the most active photosynthetic organisms and has a high density of chlorophyll. This algae improves the health and resistance of the skin. *Chlorella* extract is used in the manufacture of cosmetics and hygiene products and in pharmaceuticals due to the polysaccharides it contains (Safari et al., 2011). Algae polysaccharides, which are characterized by their high molecular weight and composed of multiple monosaccharides connected by glycosidic bonds, demonstrate

notable biological activity. Gut microbiota possesses the ability to break down *Spirulina* polysaccharides into butyrate, shortchain fatty acids, and other metabolites that can be readily absorbed and utilized by the human body (Guan et al. 2024).

2. Materials and Methods

2.1. Sampling

Sampling in order to isolate yeast and bacterial samples from different traditional dairy such as the Golpaygan; Arak and Tabriz. The collected samples were transported to the laboratory in sterile containers; Next, 100 microliters of different dilutions prepared from the samples were cultured on YGC agar and MRS agar media. The plates were heated at 30 degrees Celsius for at least 72 hours (Ren et al., 2018; Dworkin et al., 2006).

2.2. Microscopic and biochemical tests

Catalase test, oxidase test, morphological observation under the microscope, nitrate reduction test, sugar and carbohydrate fermentation, ascospore production and microbial sensitivity were carried out. For this purpose, fresh cultures of yeast and bacteria were cultivated on the considered medium and the culture medium was heated at a temperature of 30 degrees Celsius for 72 hours and then checked (Edalatian et al., 2012).

2.3. Study of the probiotic properties of the isolates

The growth of the isolates was studied at different temperatures, acidities and bile salts. For this purpose, some yeast and bacterial isolates were added to the fresh culture medium. Heat was applied at temperatures of 25, 30, 37 and 42 °C and pH 1.5, 2, 3 and 5 (Cook et al., 1969). To investigate resistance to bile salts, sodium cholate and sodium deoxycholate were added to the target media at 0.3% (Guo et al., 2006). Finally, at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 hours, a reading was taken using a spectrophotometer at a wavelength of 625 nm (Cook et al., 1969).

2.4. DNA extraction

DNA was first extracted using the Sina Gene kit. Then 1 milliliter of the cell suspension was centrifuged at 10000 g for 1 minute. After removing the supernatant, 300 microliters (Gynogen kit), 40 microliters SDS, 50 microliters Triton (Triton for fungi) and 10 microliters lysozyme (for gram-positive bacteria) were added as a second solution and incubated at 37°C for 5 minutes. Then 150 μ L of saturated saline was added as the third solution and the tube was gently inverted for 5 minutes. The tube was then centrifuged at 10000 g for 5 minutes. The supernatant was transferred to a new tube and 300 microliters of cold isopropanol was added and the tube gently inverted. The tube was then centrifuged at 6000 g for 10 minutes. At this stage, DNA deposition was observed. The supernatant was discarded and the DNA was washed by adding 300 microliters of cold 75% EtOH, the tube was inverted 2-3 times and then centrifuged at 6000 g for 5 minutes. Again, the supernatant was discarded and the tube was dried with lead and the alcohol completely evaporated. Finally, 50 microliters of distilled water were added and the sample was stored overnight at -20°C.

2.5. Identification of isolates by PCR

To ensure the presence of DNA and the accuracy of the extraction, the extracted DNA was electrophoresed using a horizontal electrophoresis system on a 1% agarose gel in TBE buffer. To perform horizontal electrophoresis, 4 microliters of each sample was mixed with 1 microliter of loading dye on adhesive tape and loaded into the gel well. The lid was placed on the container, the electrodes connected and the voltage generator set to 100 volts for 20 minutes. The gel dock was then used to view and photograph the DNA bands formed. The desired gel was placed in the gel docking device to confirm the status and correctness of the extracted plasmid sample using UV light. Finally, to perform the PCR, 7 microliters of water were first transferred to a 2 milliliter microtube. Then 1 microliter of each primer (the ITS1 and ITS4 primer sequences for yeast and the LacR primer for bacteria were prepared by Sina Clone Company) was added to the wall of the microtube. Then 1 microliter of DNA was added to the wall. Taq 2x MASTER MIX (Amplicon, Denmark) was then added and placed in the thermocycler device (Table1) (Vancov et al., 2009).

Table1. Primers used in this research (Vancov et al., 2009).

Nucleotide sequence of 18srRNA (yeast)		Tm(C°)	(bp)	Reference
Forward	AGCTGGTTGATTCTGCCAG	53	19	Forward
Reverse	TGATCCTCCGCAAGTTCAC	53	20	Reverse
Nucleotide sequence of 16srRNA (Bacteria)		Tm(C°)	(bp)	Reference
Forward	AGAGGTTCTGAGCTCAG	52	19	Forward
Reverse	ACAGCTTCCTTGTTACGATT	52	20	Reverse

2.6. Drawing a phylogenetic tree

The phylogenetic tree was constructed using 16S rRNA and 18S rRNA sequences with Mega7 software. Bootstrap 500 bacteria and 1000 yeast samples, the evolutionary distance was calculated using the Maximum Composite Likelihood method. Bacterial sequences were analyzed as 9 nucleotides and yeast sequences as

10 nucleotides. The scale of difference is 0.05 for bacterial samples and 0.1 for yeast samples.

2.7. Extraction of polysaccharide from *Lentinula edodes* and *Ganoderma lucidum*

To extract the polysaccharide, after culturing the strains in liquid culture medium and obtaining a high density of mycelium and lyophilization of the mycelium and fruiting

organ to extract the lentinan polysaccharide, it was done according to the method of Ataollahi et al. (Ataollahi et al., 2021), up to three layers were formed. (the white middle layer contains protein, the lower layer contains the extracted polysaccharide lentinan) by taking two outer layers, the remaining liquid is deproteinized with three volumes of pure ethanol, dialyzed and vigorously stirred for 12 hours at 4 degrees Centigrade was kept and crude lentinan was obtained by centrifugation at 2500 revolutions in 15 minutes (Ren et al., 2018).

Purification of extracted polysaccharide: DEAE-Sephadex A ion exchange chromatography column was used for further purification of the extracted lentinan polysaccharide (Sadasivam et al., 2005).

2.9. Preparation of spirulina and chlorella polysaccharides

Spirulina algae powder, which was purchased from domestic sources (Iran Research Institute of Chemistry and Chemical Engineering), to extract cellulose from *spirulina* algae, it was first purified by chemical treatment and the cellulose free from protein, fat, extractive substances and other impurities from algae was obtained. The appearance of the suspension of algae and chemicals before and after three stages of chemical treatment is shown in Figure 1 (Moradpour et al., 2018).

Chlorella algae powder was purchased from domestic sources (Iran Institute of Chemistry and Chemical Engineering). Soxhlet method was used to extract polysaccharide from *Chlorella* algae (Safari et al., 2011).

2.10. Measurement of total extracted polysaccharide concentration

To determine the concentration of extracted polysaccharide, the phenol-sulphuric acid method was used, among other colorimetric methods. Fifty microliters of the sample were added to each well of the 96-well microplate, and 150 microliters of concentrated sulphuric acid was quickly added to each well and mixed for 30 minutes. Then 50 microliters of 5% phenol in added water was heated for 5 minutes in a hot water bath at a temperature of 90 degrees Celsius, and after cooling a little, it was again placed in another water bath equivalent to

the ambient temperature for 5 minutes, plus the standard glucose was also used, and finally the microplate was placed in the Eliza rider device and the ultraviolet absorbance was read at 490 nm (Masuko et al., 2005).

2.11. Investigation of the structural characteristics of the extracted polysaccharide by FT-IR method

The structure of the extracted polysaccharide was identified using an infrared Fourier transform instrument. The infrared Fourier transform spectrum from 4000 to 650 $[\text{cm}]^{-1}$ with a resolution of $[\text{cm}]^{-1}$ 1 was recorded using a Bruker Tensor 27 spectrometer equipped with an ATR system (Yang et al., 2011).

2.12. The effect of resistance to acid and enzymatic digestion of the extracted polysaccharide

In order to test the resistance to digestion of the extracted polysaccharide, the simulation of digestive juices was carried out according to the method of Jain et al. To simulate the digestive juices of the stomach, sodium chloride and hydrochloric acid were used in specific proportions and their pH was adjusted to 1.2 ± 0.5 (buffer 1). To simulate the intestine, potassium dihydrogen phosphate and sodium carbonate were used in specific proportions and the pH was adjusted to 7.4 (buffer 2). To simulate the mixture of stomach and intestine, buffers 1 and 2 were mixed in a ratio of 39:61 and the pH was adjusted to 4.5. The extracted polysaccharide and inulin were added to the prepared buffers and kept in a shaker incubator at a speed of 100 g and a temperature of 37°C. After centrifugation for one hour, a sample was taken from the supernatant buffer to measure free sugars. The 3,5-dinitrosalicylic acid (DNS) method was used to measure free sugars. The percentage of hydrolysis was reported based on the amount of sugar released relative to the total sugar (Jain et al., 2007; Azmi et al., 2012).

2.13. Technological characteristics

The water holding capacity and lipid adsorption capacity of the extracted polysaccharide were investigated and inulin was

measured as a control prebiotic according to the method of Carvalho et al. (2009). In this method, 30 milliliters of distilled water were added to one gram of sample and stirred well to determine the WHC. The desired mixture was kept at room temperature for one hour and then centrifuged at 12000 g for 20 minutes. The supernatant was discarded and the residue weighed. WHC is expressed as the ratio of water weight to sample weight. For the determination of LAC, 3 g of the sample was added to 18 ml of sunflower oil and left overnight at room temperature. The desired mixture was centrifuged at 1500 g for 10 min, the excess oil was discarded and the residue was weighed. LAC was expressed as the ratio of oil weight to sample weight (Fan et al., 2010).

2.14. Assay of antioxidant properties

To test the antioxidant properties of the extracted polysaccharide, 0.2 g of polysaccharide was mixed with 5 ml of methanol and stirred vigorously on a shaker incubator for 3 hours. The mixture was then centrifuged at 3000g for 20 minutes and the supernatant was used to test the antioxidant activity. To compare the antioxidant activity of polysaccharides extracted with inulin as a prebiotic standard, the same conditions were applied to inulin. In addition, 1000 micromolar ascorbic acid solution was used as a positive control. To investigate the effect of concentration on the antioxidant activity of the extracted polysaccharide, the concentration range of 0.005-0.04 g/ml sample was prepared and then stirred and centrifuged as before. The DPPH method was used to check the antioxidant activity. To perform this test, 500 microliters of methanolic extract was rapidly added to 5 ml of methanolic solution of 0.1 mM DPPH and vigorously mixed. The resulting mixture was placed in the dark for 30 min at room temperature and the absorbance was read at 517 nm (Norajit et al., 2010). The absorbance of the DPPH methanolic solution was also read at 517 nm and the amount of antioxidant activity was calculated using the following equation (Almasi et al., 2014):

$$\text{Scavenging activity\%} = \frac{(\text{Abs}]_{\text{blank}} - [\text{Abs}]_{\text{sample}})}{[\text{Abs}]_{\text{blank}} * 100}$$

Abs blank: Absorbance of methanolic DPPH solution without polysaccharide sample

Abs ample: Absorbance of methanolic DPPH solution with polysaccharide sample

2.15. Investigating the effect of the extracted polysaccharide on the growth of probiotic bacteria

A specific strain of Lactobacillus was selected as an indicator bacterium for growth in the presence of prebiotics. The base medium was chosen to add the prebiotic composition of the MRS medium without sugar, and its components were added according to the instructions of the said medium. Then, 2% (w/v) extracted polysaccharide was added to the target medium to study the bacterial growth in the presence of the prebiotic compound. To compare the growth of probiotic bacteria in the presence of polysaccharide isolated from media containing 2% glucose and 2% inulin, a commercial prebiotic was used as an index. Activated Lactobacillus bacteria were added to the said media at a ratio of 2% (v/v). Samples were taken from the above environments at 0, 24, 48 and 72 h and were cultured and counted according to the method of Miles Mizera (1938) (Miles et al., 1938).

2.20. Investigating the effects of probiotics

2.20.1. Surveying the prebiotic properties of polysaccharides on the growth of isolated yeast and bacteria

Inulin was used as a commercial and indicator prebiotic. The effects of synergism of extracted polysaccharide compounds on the growth rate of isolated probiotics were studied and 1% (v/v) was added to the said media. Bacteria and yeast isolates were added at 1%. Samples were then taken after 2, 4, 6, 8, 10, 12, 24, 48 and 72 hours and read using a spectrophotometer at a wavelength of 620 nm.

2.20.2. Investigation of lactic acid production

To measure lactate from diagnostic enzyme kits, the lactate assay kit was measured according to the manufacturer's instructions using appropriate dilutions. Cell growth (OD 546) and at 37°C was also evaluated over time. The lactate oxidase enzyme reacts lactate to

pyruvate and hydrogen peroxide in the presence of peroxidase with 4-aminoantipyrine and TBHB to produce the red compound quinonimine. The increase in color produced is proportional to the concentration of lactate in the sample. The lactate concentration can be calculated from the following formula (Rahman et al., 2015).

(Absorbance difference sample) / (Absorbance difference standard) $\times$ Concentration standard = Lactate concentration

Lactate (mg/dl) $\times$ 0.11 = Lactate (mmol/l)

All experiments were repeated three times to be sure.



Figure 1. (A) suspension and chemicals, before and (B) after three stages of chemical treatment

Chlorella algae powder was purchased from domestic sources (Iran Institute of Chemistry and Chemical Engineering). Soxhlet method was used to extract polysaccharide from *Chlorella* algae (Safari et al., 2011).

3. Results

3.1. Sampling and isolation of yeast and bacterial isolates

After sampling and culturing the samples on MRS, PDA, YGC and Chrom agar media, 10 yeast isolates and 10 bacterial isolates were obtained from the water and soil samples, of which only two bacterial strains and two yeast strains were related to probiotic strains. Yeast isolates grew better on YGC agar, PDA and bacterial isolates on MRS agar. After successive cultures, the isolates were isolated as single colonies.

3.2. The results of biochemical tests and confirmatory tests

The results of biochemical tests including the fermentation of sugars 10 bacterial isolates and 10 fungal isolates isolated from food sources are shown in Tables 2 and 3.

3.3. Antibioqram results

The antibiotic sensitivity results of yeast and bacterial isolates using disk diffusion method and based on the CLSI table were shown in Table 4. These results indicated that isolates A1 and A9 were resistant to fluconazole, sensitive to ketoconazole and Amphotericin B. B1 and B5 isolates were resistant to Ceftazidime and Tetracycline and sensitive to Clindamycin, Chloramphenicol, Gentamicin, and Levofloxacin. These results agreed with the results of Tumu. et al. that most of the *lactic acid* bacteria were resistant to tetracycline (Thumu et al., 2012). *Lactic acid* bacteria isolates were also resistant to Ceftazidime antibiotic. It is a natural feature of *lactic acid* bacteria to have inherent and acquired resistance (Ammor et al., 2007).

3.4. Acid, temperature and bile salt resistance testing of yeast isolates

Resistance to pH and bile is a prerequisite for probiotics to survive and grow in the digestive tract. The pH of digestive juice varies between 1.5 and 3.5 depending on feeding time, growth

stage and animal species. The growth of yeast isolates at pH ~5.3 was initially exponential, but after 24 hours the growth rate decreased significantly. The lowest growth was also observed at pH ~2.1.5. At the temperature of 25°C and relatively 30°C, they had better growth and adaptation than at the temperature of 42°C and 37°C. As probiotics are usually administered orally, they must be able to survive during passage through the rumen and small intestine. Therefore, resistance to the low pH of the digestive juice in the rumen and bile salts in the small intestine is an important criterion for their selection (Ouwehand et al., 1999) Resistance to bile salts is a prerequisite for bacterial

colonization and metabolic activity in the host small intestine (Hood et al., 1988). The average concentration of bile is about 0.3%, which can reach 2% during the first hours of digestion. In this study, bile tolerance was investigated in yeast strains. The results in terms of growth in the presence of bile salt in the figure 2 showed that the isolates in question had the ability to grow in the presence of this salt, but their growth was reduced (Lin et al., 2007).

Table 2. Fermentation results of carbohydrates of yeast isolates

	Glucose	Sucrose	Arabinose	Maltose	Mannitol	Sorbitol	Dextrin
A1	+	-	-	-	+	+	-
A2	+	-	-	-	+	+	-
A3	+	+	-	-	+	-	-
A4	+	+	-	-	+	-	-
A5	-	+	-	+	-	-	-
A6	+	-	-	+	-	-	+
A7	+	+	-	-	+	-	-
A8	+	+	-	-	+	-	-
A9	+	-	-	-	+	+	-
A10	+	+	+	-	-	-	-

Table 3. The results of fermentation of carbohydrates of bacterial isolates

	Glucose	Sucrose	Arabinose	Maltose	Mannitol	Sorbitol	Dextrin
B1	+	+	-	-	+	-	-
B2	+	+	-	-	+	-	-
B3	+	+	+	+	+	+	+
B4	+	-	+	-	+	-	+
B5	+	+	-	-	+	-	-
B6	-	-	-	-	+	+	+
B7	-	-	-	-	+	-	-
B8	-	+	-	-	+	-	-
B9	-	+	+	-	+	+	+
B10	+	+	-	+	-	-	-

Table 4. Antibiotic sensitivity test of obtained isolates

Antibiotic name	A1	A9	B1	B4
Ketoconazole	S	S	s	.
Amphotericin B	R	S	.	.
Fluconazole	S	S	.	.
Tetracycline	.	.	R	S
Clindamycin	.	.	S	S
Chloramphenicol	.	.	S	S
Gentamycin	.	.	S	S
Levofloxacin	.	.	S	S
Ceftazidime	.	.	R	S
Streptomycin	.	.	S	R
Vancomycin	.	.	S	S

R: resistant, S: sensitivity

3.5. Acid, temperature and bile salt resistance test on bacterial isolates

In bacterial isolates, the best growth is at pH ~5. At pH ~3 there was good growth in the first 5 hours, but after 24 hours there was a significant decrease, and after a few hours there was significant growth again, and then a decrease in growth was observed again. pH ~2 had good growth at first, but then a decrease in growth was observed. The isolated lactic acid bacteria die if they are not resistant to acidic conditions (Erkkilä et al., 2000). It had the best adaptation at 37°C, but due to the exponential nature of the temperature at 42°C, it did not grow optimally. Resistance to bile salts is a prerequisite for bacterial colonization and metabolic activity in the host small intestine (Naseeb et al., 2017). In this way, lactobacilli can easily reach the small intestine and form a colony that balances the intestinal microflora. The average concentration of bile is about 0.3%, which can reach 2% during the first hours of digestion. In this study, the bile tolerance of the strains was investigated. The results in terms of growth in the presence of bile salt showed that the bacterial isolates in question had the ability to grow in the presence of this salt, but their growth was reduced. Resistance to bile salts varies between strains and even between species. Some bile salt resistance in some strains depends on the enzyme BSH (bile salt hydrolyzing enzyme), which causes the hydrolysis of conjugated bile salts and reduces the hydrolysis of the toxicity of activated bile salts (Lin et al., 2007). The results are shown in figure 3.

3.6. Molecular identification and phylogenetic tree construction

The 18sRNA sequencing of isolates A1 and A9 are closely related to *Pichia fermentans* strain 1691, *Saccharomyces cerevisiae* strain 1137 and the 16sRNA sequencing of isolates B1 and B5 are closely related to *Lactobacillus corriformis* strain 1197 and *Lactobacillus acidophilus* strain 3103. The phylogeny of these strains was also inferred by maximum likelihood.

3.7. Examination of the extracted polysaccharide by acid and enzymatic digestion

The DNS (dinitro salicylic acid) test is used to estimate the amount of reducing sugars present in the environment. Reducing sugars have a free carbonyl group (a large group of organic compounds in which a carbon atom is linked to an oxygen atom by a double bond) which can reduce many reagents. All monosaccharides and many disaccharides are reducing sugars. When the alkaline solution of 3,5-dinitrosalicylic acid reacts with a reducing sugar (e.g. glucose, lactose, etc.), it converts to 3-amino-5-nitrosalicylic acid and the color of the reagent changes from yellow to orange or red, i.e. The color change depends on the concentration of the reducing sugar. The results of measuring the amount of reducing sugars by DNS method are shown in Table 5.

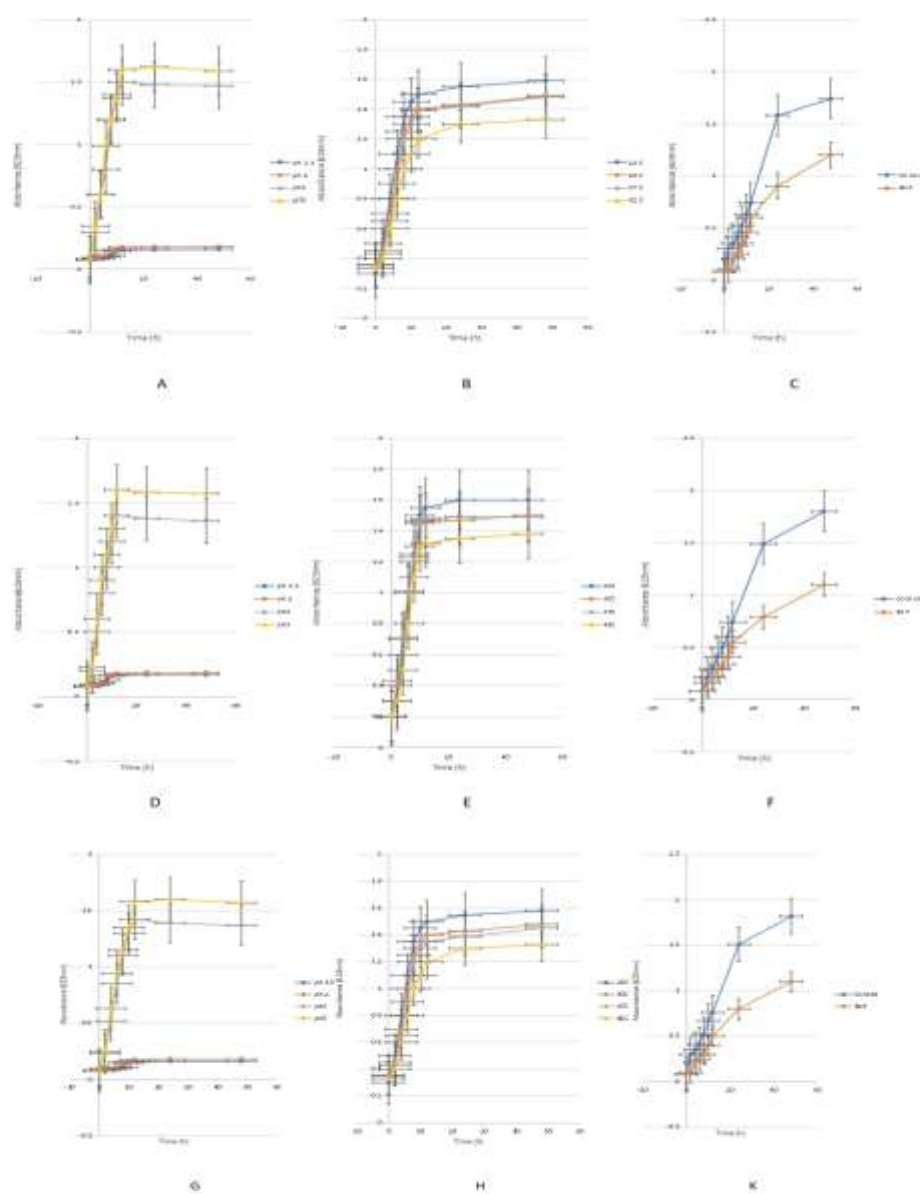


Figure 2. A, B, C Investigation of pH, temperature and bile salt resistance in standard yeast sample, D, E, F Investigation of pH, temperature and bile salt resistance in A1 sample and G, H, K Investigation of pH, temperature and bile salt resistance in the sample A9

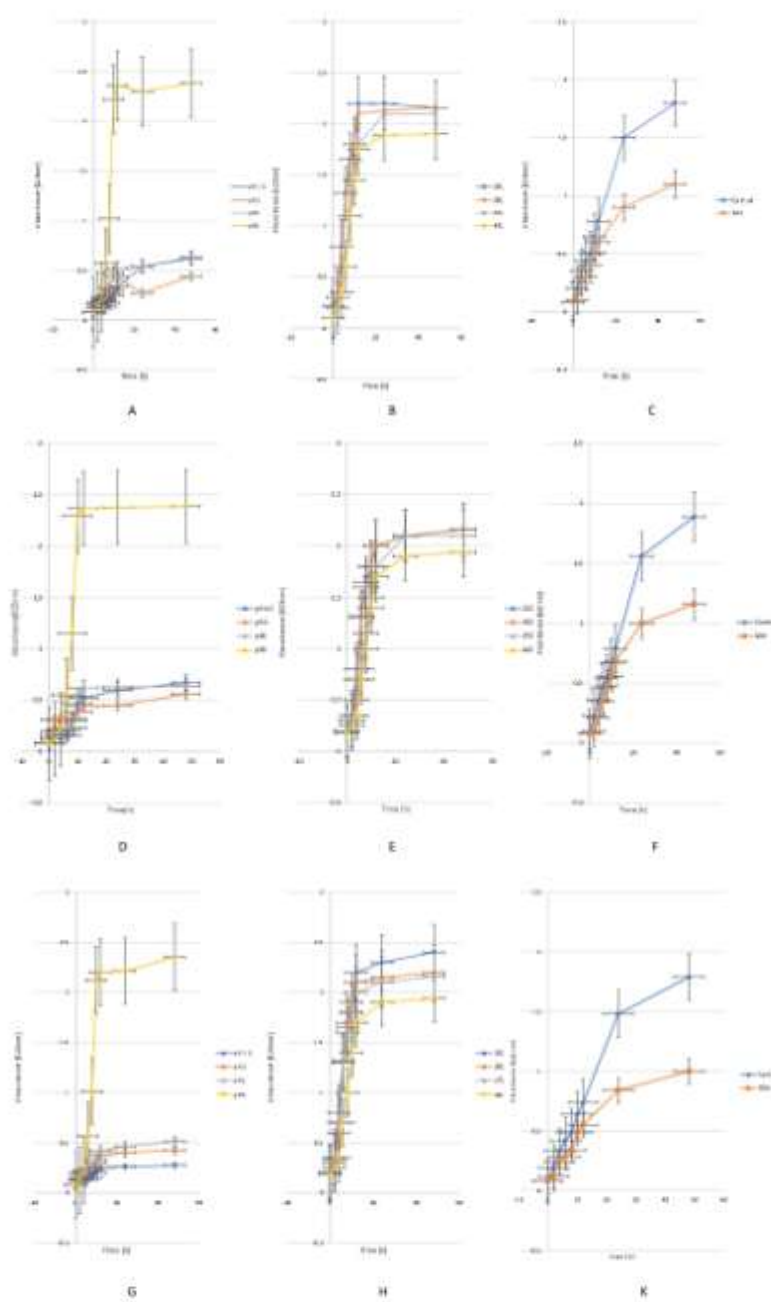


Figure 3. A, B, C Check pH, temperature and bile salt resistance in the standard bacteria sample, D, E, F Check pH, temperature and bile salt resistance in sample B1 and G, H, K Check pH, temperature and bile salt resistance in the sample B5

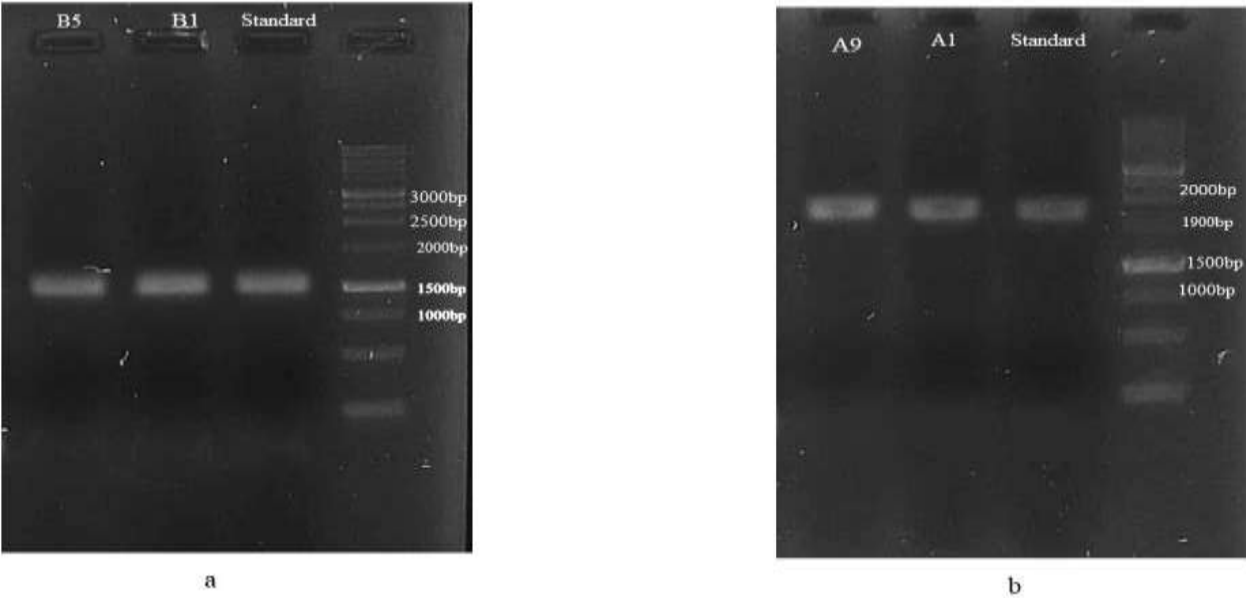


Figure 4. a) PCR of bacterial isolate, b) PCR of yeast isolate

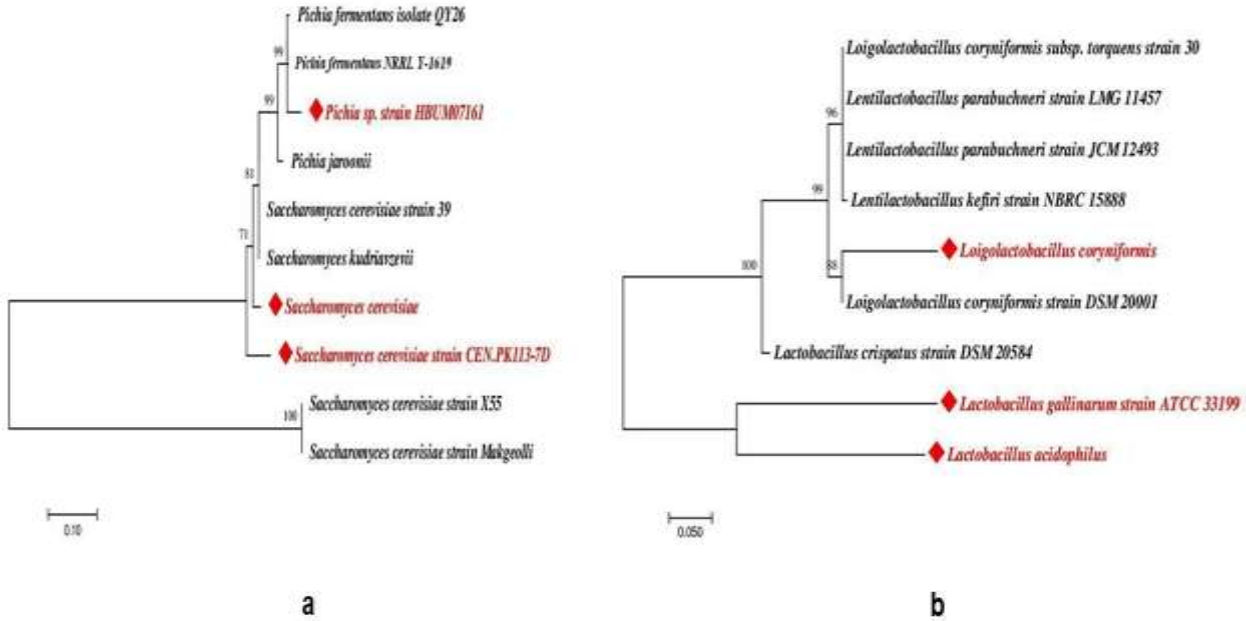


Figure 5. a) Phylogeny tree of yeast isolates with Maximum likelihood method, b) Phylogeny tree of bacterial isolates with Maximum likelihood method

Table 5. The results of measuring the amount of reducing sugars by DNS method

	Absorption standard sample / 543 nm	Absorption samples / 543 nm
<i>Inulin</i>	0.2	0.45
<i>Ganoderma</i>	0.15	0.3
<i>Lentinula</i>	0.12	0.23
<i>Spirulina</i>	0.13	0.39
<i>Chlorella</i>	0.11	0.28

3.8. Study of the technological properties of the extracted polysaccharides

In order to check the technological applicability of the isolated polysaccharides, the characteristics of water retention and fat absorption were measured. As can be seen, the water retention capacity of *lentinula* polysaccharide is much higher than that of inulin. This issue is very important both technologically and physiologically. From a technological point of view, compounds with the ability to retain and absorb water can lead to an increase in viscosity and prevent water from returning. Compounds with a high-water retention capacity can help reduce the rate of absorption of substances by increasing the viscosity of the food mass in the digestive tract, increasing the volume of feces and increasing the frequency of defecation. This property is effective in controlling weight and improving health, particularly in preventing the onset of cardiovascular disease and diabetes (Azmi et al., 2012; Elleuch et al., 2011). Fat absorbency refers to the ability of the polysaccharide network to absorb fat. Physiologically, due to their ability to absorb fat, they can help reduce the absorption of fat from food into the blood. As can be seen, the ability of the polysaccharides extracted from *Lentinula* to absorb fat is greater than that of inulin. Due to the characteristic of fat and water absorption, isolated polysaccharides, their prebiotic properties can be a suitable option as a bioactive polysaccharide and can compete with inulin for applications. The results Water retention capacity and Fat absorption capacity by polysaccharides are shown in Table 6 a and b.

3.9. Study of the antioxidant property of the extracted polysaccharide

Diphenyl picrylhydrazyl is a stable free radical. In this method, the ability of the available extracts to release hydrogen atoms or electrons is measured by the amount of color change from purple solution to yellow solution. Due to the presence of a single electron in the structure of DPPH, this radical has a good absorption at the wavelength of 517 nm and when it is in the presence of an antioxidant compound that has free radical scavenging activity, its color is removed and the absorption at the wavelength of 517 nm is reduced. This is an expression of the activity of the antioxidant sample. Among the 4 types of polysaccharides tested and an indicator called inulin, the highest DPPH free radical scavenging activity is associated with *Lentinula*, *Spirulina*, *Chlorella* and *Ganoderma* polysaccharides respectively. Inulin also has the highest radical scavenging rate as an indicator. The absorption of methanolic DPPH solution with polysaccharide sample is shown in the table 7.

3.10. Analysis of the extracted polysaccharide structure by infrared Fourier transform method

Infrared Fourier Transform method was used to check the structural characteristics of the extracted polysaccharide. The corresponding results are shown. The infrared Fourier transform method is usually used in polysaccharides to check the type of glycosidic linkages, the type of monosaccharides and the functional group (Yang and Zhang, 2009).

FT-IR obtained from *Spirulina* algae, *Chlorella* algae, combination of polysaccharides extracted from *Chlorella* and *Spirulina* algae,

Lentinula fungus, *Ganoderma* fungus, combination of polysaccharides extracted from *Ganoderma* and *Lentinula*, combination of polysaccharides extracted from *Ganoderma* fungi, *Lentinula* and *chlorella* algae and *spirulina* shows before and after chemotherapy treatment. The results showed that the produced sample was very consistent with the samples obtained from another research related to FT-IR test. The structure of the links in the polysaccharide extracted from fungi and algae is shown in the figure 6.

3.11. Study of the effect of the extracted polysaccharide on the growth of probiotic bacteria

This study investigated the effect of polysaccharides on a standard probiotic bacterium (*Lactobacillus acidophilus*). Inulin was used as a probiotic indicator and glucose as a control. According to the results obtained, it was found that the presence of polysaccharide has a positive effect on the growth of standard bacteria. As in figure 7 has been shown that inulin and glucose gave the most growth, followed by 4 polysaccharides, 3 polysaccharides and finally the single polysaccharide combination.

3.12. The results of investigating the growth of probiotics in the presence of prebiotics

In order to investigate the growth of probiotics in the presence of prebiotics, the factorial method was used, so that the synergistic effect of each of the components in the experiment was investigated in single, double, triple, etc. Due to the existence of four variables, there were fifteen (Masuko et al., 2005) general modes for their combination. According to graphs 14 -19, in the composition of a type of polysaccharide, which contains yeast + *Ganoderma* and bacteria + *Ganoderma*, probiotic growth was low compared to the rest of the combinations. In the combination of two types of polysaccharides, the tube containing yeast + *Lentinula* + *chlorella* and bacteria + *Lentinula* + *chlorella*, the growth was relatively better than before. In the combination of three types of polysaccharides, yeast + *Ganoderma* + *Lentinula* + *spirulina* and bacteria + *Lentinula* + *chlorella* + *spirulina* have grown the most compared to the previous cases. The tube containing inulin has grown more growth, 3 polysaccharides, and finally the single polysaccharide combination. The results are shown in figure 8.

Table 6. A) Water retention capacity by polysaccharides, B) Fat absorption capacity by polysaccharides

A		
	Water retention capacity by polysaccharides / gram/After	Water retention capacity by polysaccharides /gram/Before
<i>Inulin</i>	1	1.5
<i>Ganoderma</i>	1	1.65
<i>Lentinula</i>	1	1.7
<i>Spirulina</i>	1	1.68
<i>Chlorella</i>	1	1.62
B		
	Fat retention capacity by polysaccharides/ gram/After	Fat retention capacity by polysaccharides/ gram/After
<i>Inulin</i>	3	4.0
<i>Ganoderma</i>	3	3.9
<i>Lentinula</i>	3	4.2
<i>Spirulina</i>	3	3.5
<i>Chlorella</i>	3	3.6

Table 7. Absorption of DPPH methanolic solution with polysaccharide sample

	% antioxidant activity	Adsorption of DPPH
<i>Inulin</i>	59	0.0815
<i>Ganoderma</i>	40.5	0.1190
<i>Lentinula</i>	41.65	0.1167
<i>Spirulina</i>	41.4	0.1172
<i>Chlorella</i>	41	0.1180

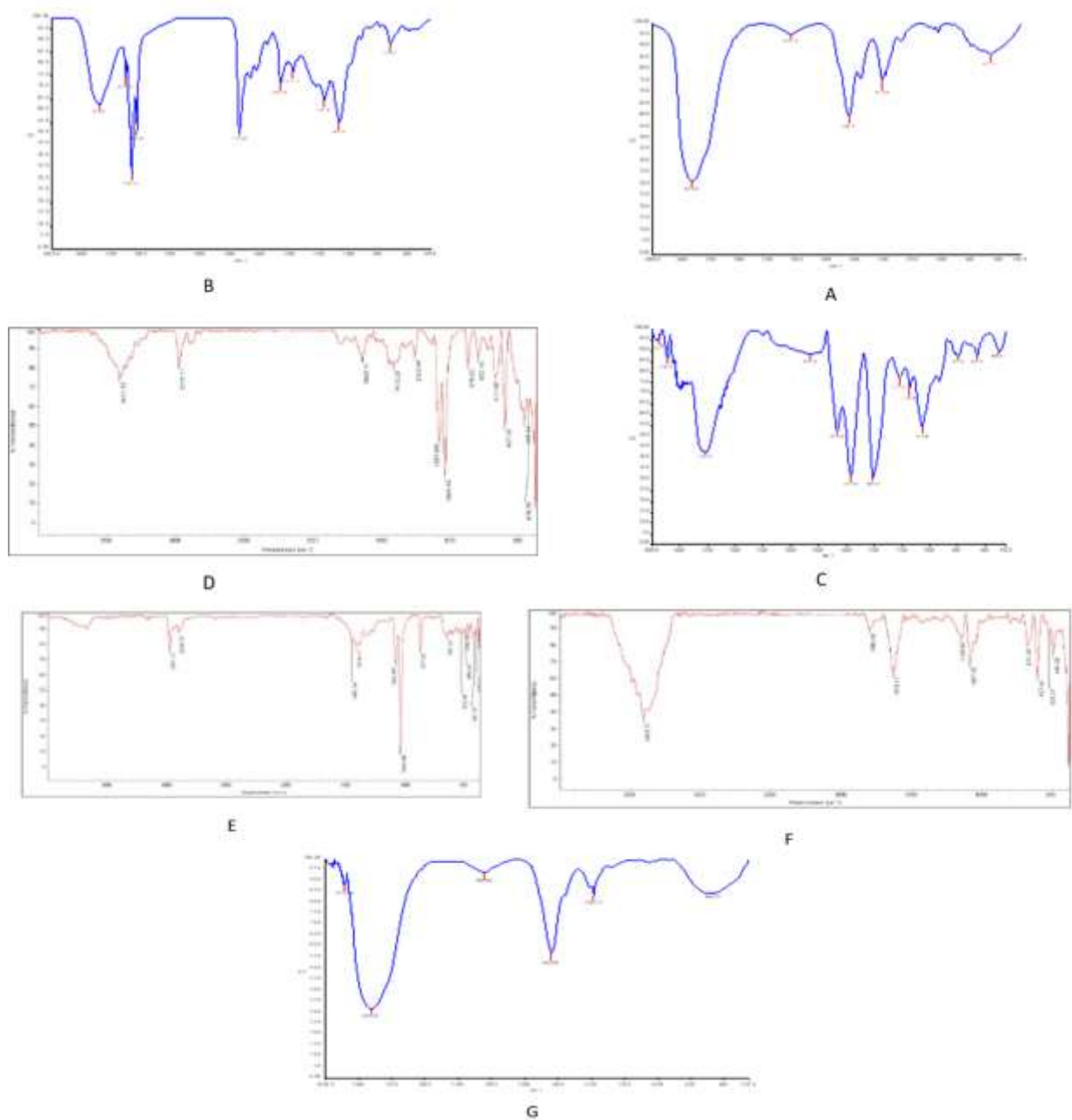


Figure 6. Analysis of the extracted polysaccharide structure by infrared Fourier transform method. A. peaks of the polysaccharide structure extracted from *Spirulina*, B. *Chlorella*, C. *Chlorella* and *Spirulina*, D. *Ganoderma* and *Lentinula*, E. *Ganoderma*, F. *Lentinula*, G. *Ganoderma*, *Lentinula* and *Chlorella* and *Spirulina*

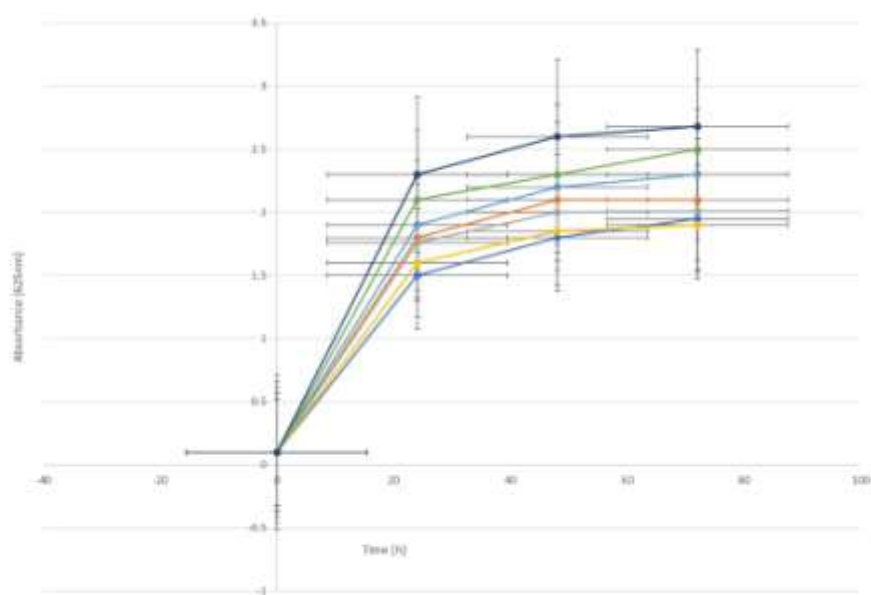
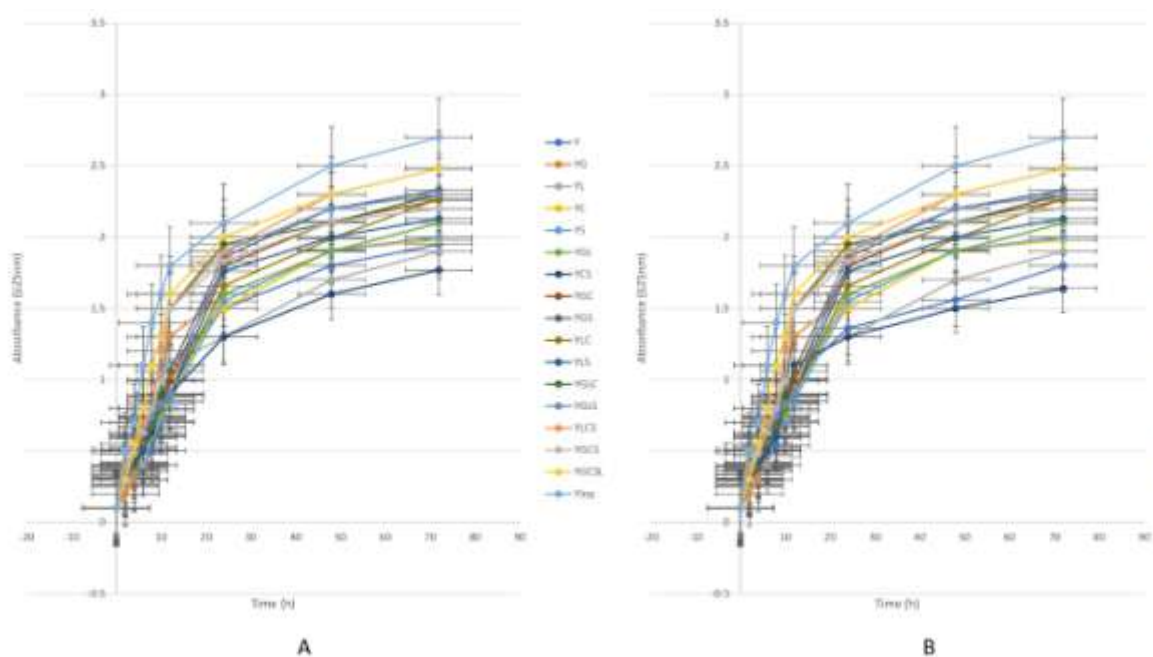


Figure 7. Investigating the effect of the extracted polysaccharide on the growth of probiotic bacteria



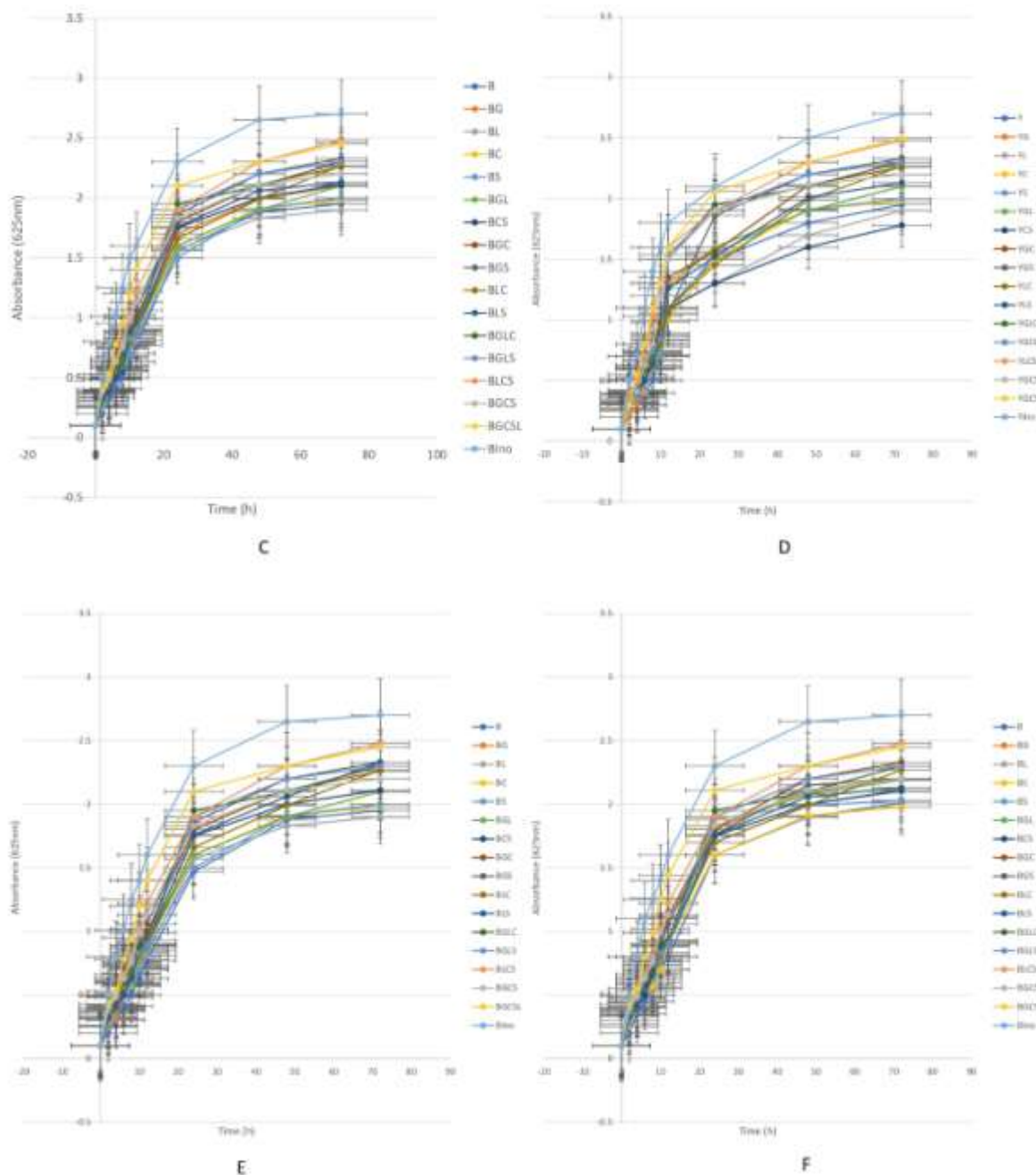


Figure 8. A. The effect of prebiotics on the growth of standard yeast (*Saccharomyces cerevisiae*), B. A1 yeast (*Pichia fermentans*), C. standard bacteria (*Lactobacillus acidophilus*), D. yeast A9 (*Saccharomyces cerevisiae* strain CEN.PK113-7D), E. bacteria B5 (*Lactobacillus coryniformis*), F. bacteria B1 (strain NWAUFU1197 *Lactobacillus coryniformis*)

3.13. Results of lactate acid production in the presence of prebiotics

Inulin was used as a commercial probiotic. As in the previous test, inulin produced the most lactate acid produced by the bacteria. Then the combination of 4 polysaccharides ranks first in lactate production. This is followed by a

combination of triple, double and single polysaccharides. When comparing yeast and bacteria, the amount of lactate produced was higher in bacteria than in yeast. The results are shown in table 8.

3.14. Statistical Analysis

The experiment's gathered information underwent statistical analysis, where the samples

were grouped using a two-way ANOVA test. The data obtained from the experiment were subjected to statistical analysis are shown in the figure 9 and table 9.

Table 8. Investigation of lactate concentration for yeast isolates density

	The amount of lactate concentration (standard)	The amount of lactate concentration (A1)	The amount of lactate concentration (A9)
Y	55	55	55
YG	61	62	61.5
YL	61.5	62	61.25
YC	60	60.5	59.5
YS	62	61	61.5
YGL	68	67	70
YCS	66	66	65
YGC	70	71	72
YGS	72	78	77
YLC	82	85	84
YLS	85	85	78
YGLC	90	89.5	89
YGLS	89.5	91	89
YLCS	85	95	85
YGCS	88.5	88	88
YGCSL	109	105	110
YIno	110	110	110

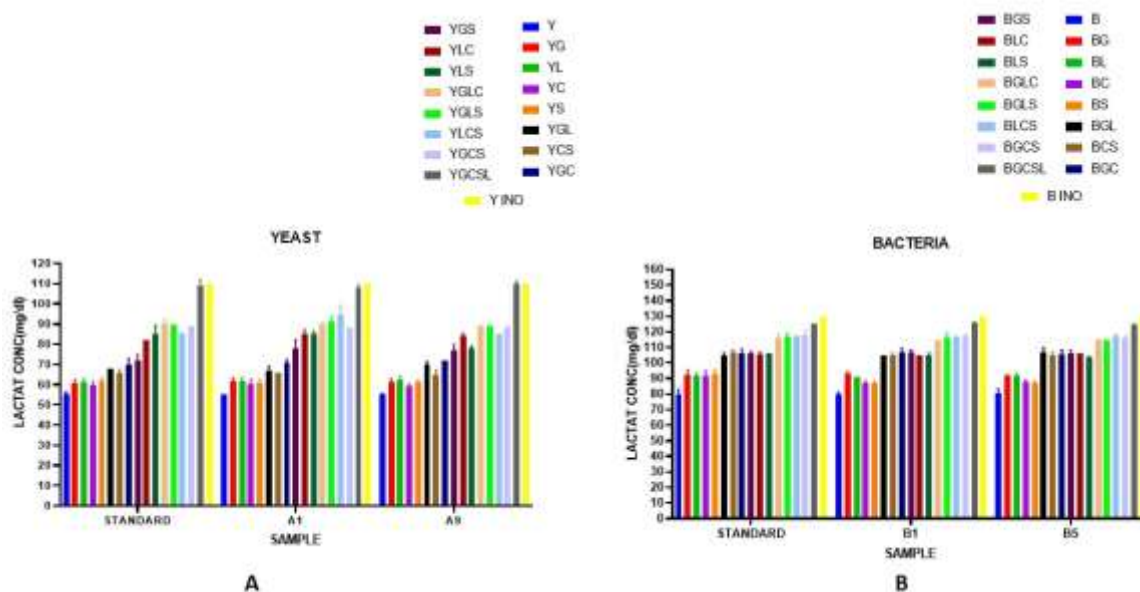


Figure 9. The amount of lactate production in standard samples, A1 and A9 with Graph Pad software, B) B1 and B5 with Graph Pad software

Table 9. Lactate production rate in yeast sample with 2-way ANOVA test, B) Lactate production rate in bacterial sample with 2-way ANOVA test

A				
ANOVA table	% of total variation	P value	P value summary	Significant?
LACTAT CONC(SAMPLE)	0.9175	<0.0001	****	YES
LACTAT CONC(SAMPLE)	0.9175	<0.0001	****	YES
B				
ANOVA table	% of total variation	P value	P value summary	Significant?
LACTAT CONC(SAMPLE)	0.4979	0.0004	***	YES
LACTAT CONC(STANDARD)	0.1239	<0.0001	****	YES

4. Discussion

In recent years, studies have extensively focused on yeasts that have been isolated from different environments. However, most studies have concentrated on yeasts related to food origins, with little attention given to environmental species (Naseeb et al., 2017). Polysaccharides, which are sourced from algae and fungi, are beneficial for their probiotic properties and are commonly used as a food source and supplement. Therefore, they are a suitable replacement for the limited availability of animal and food resources. To accurately determine the identification and isolation of yeasts, it is essential to consider the physical-chemical characteristics of the environment as a vital ecological factor in determining their habitat. These characteristics, combined with nutritional features, contribute to the diverse range of habitats in which yeasts can thrive (Troncoso et al., 2017). The study identified and analyzed four native isolates - A1 and A9, alongside *Pichia* sp. strain HBUM07161, *Saccharomyces cerevisiae* strain CEN.PK113-7D and bacterial isolates B1 and B5 - specifically *Lactobacillus gallinarum* strain ATCC 33199 and *Loigolactobacillus coryniformis*. All strains possess the requisite probiotic properties to be utilized as a probiotic agent. Owing to their ability to grow at the body temperature of 37 degrees Celsius, withstand a

pH of 1.5, and resist bile salt, these isolates may serve as a potential product. This can be confirmed by conducting further tests to determine toxin production or non-production in the host's body. Furthermore, the introduction of indigenous probiotics is recommended. The investigated isolates grew proficiently within an acidity range of 1 to 5.5 and a temperature range of 25 to 42 degrees Celsius. These findings were consistent with Shetaia et al.'s (2016) research (Shetaia et al., 2016), indicating that the isolated strains possess the capacity to withstand extreme conditions (Nasir et al., 2021). It is evident that the fraction of polysaccharide hydrolysis obtained during the initial phase of digestion in the stomach is significantly lower than that of inulin. There is a notable variance of 1% during the subsequent phase of digestion. During the three stages of testing, the mixture of stomach and intestine was combined with the intestine in the presence of alpha-amylase enzyme. The resultant percentage of hydrolysis obtained for both extracted polysaccharide and inulin samples was negligible and almost identical. It should be acknowledged that a fundamental requirement to establish a compound's prebiotic nature is its ability to resist degradation by digestive juices and enzymes. To achieve the desired effect, the prebiotic composition needs to pass through the upper segment of the healthy digestive system and ultimately reach the large intestine, where probiotic microorganisms put it

to use. The findings in this section demonstrate that the isolated polysaccharide proved more resilient than inulin. The studies conducted by researchers in this field illustrate that the digestive resistance of polysaccharides varies greatly due to structural differences from different sources. For instance, according to Firdaus et al. (2012), the bamboo-derived polysaccharide demonstrated over 99% resistance to digestion (Firdaus et al., 2012). Wichienchot et al. (2010) reported that the hydrolysis percentage of the polysaccharide extracted from Pitaya against alpha-amylase enzyme was approximately 11%. The study also revealed that inulin's hydrolysis percentage against acidic environments was roughly 55% (Wichienchot et al., 2010). To examine the impact of extracted polysaccharides on the growth of probiotic bacteria (*Lactobacillus acidophilus*) and their ability to metabolize this compound, sugar-free MRS medium with 2% extracted polysaccharide was chosen. Additionally, to contrast the microbe's behavior, Sugar-free MRS media with 2% inulin and sugar-free MRS media with 2% glucose were employed. Over the course of 72 hours, the pH levels of the samples were measured every 24 hours. The extracted polysaccharide was found to stimulate bacterial growth. Additionally, the microbe's viability increased for a full 72 hours. However, in the medium containing glucose, the microbe population experienced a sharp decrease just after 24 hours. Furthermore, the microbe's behavior in the medium containing the extracted polysaccharide resembled that of the medium containing inulin (a commercial prebiotic). Various studies have examined the growth-stimulating effect of a compound to demonstrate its prebiotic ability over the course of 24 or 48 hours. When exploring the impact of polysaccharides extracted from agar and alginate, only the growth-stimulating effect on *Bifidobacterium* was assessed after 24 hours (Ramnani et al., 2012). Wichienchot et al. (2010) examined the growth-stimulating effect of a specific type of oligosaccharide obtained from Pitaya on *Lactobacillus delbrueckii* species, after 48 hours (Wichienchot et al., 2010). However, Firdaus et al. (2012) investigated the impact of polysaccharides derived from bamboo plants on probiotic bacteria during a 72-hour period. The study reported an enhanced survival rate of over 48

hours (Firdaus et al., 2017). Spirulina polysaccharides have the potential to regulate gut microbiota and metabolites, thereby affecting the occurrence and development of diseases. However, the interaction between Spirulina polysaccharides and gut microbiota is still in its early stages. Further research is needed to clarify the structure–activity relationship between Spirulina polysaccharides and gut microbiota, identify the main active substances of Spirulina polysaccharides that regulate gut microbiota, elucidate the mechanism by which Spirulina polysaccharides regulate intestinal metabolites, and investigate the absorption and utilization of Spirulina polysaccharides in the intestinal tract. Changes in gut microbiota are closely associated with the development of diseases. A better understanding of the structure and biological activity of Spirulina polysaccharides will facilitate their clinical application, help maintain a stable intestinal microenvironment, reduce disease risks, and promote overall health (Guan et al. 2024).

Studies by various researchers show that the structural differences and digestion resistance of different extracted compounds, as well as the constituents and buffering capacity of investigated culture media, and the behavior and capability of studied probiotic species vary. Due to differences in metabolism, variations in the growth period under investigation, and fluctuations in the concentration of the prebiotic compound analyzed, no definitive pattern can be established for comparing a recently extracted compound with earlier research. Therefore, the present study employed inulin as a commercial index prebiotic to compare characteristics of the newly extracted compound at every stage. This was done to provide an unambiguous reference for the comparison of the compound's characteristics. The study results indicate that the isolated polysaccharides possess numerous desirable features making them an appropriate option for enhancing food quality and promoting health benefits. Further studies using both animal and human models are necessary to confirm these findings.

Contribution of authors

Conceptualization; data management; formal analysis; investigation; Methodology; Dr. M. Larypoor, Dr. M. Emtiazjoo, Mr. A. Rezaei

Project Management; References; software; supervision; Validation; Dr. M. Larypoor

Visualization; Writing - original draft writing - review and editing; Dr. M. Larypoor, Mr. A. Rezaei

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Conflict of interest

There is no conflict of interest between the authors

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