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Effect of Polysaccharides Obtained from Plantago major L. leaves on Lactobacillus bulgaricus L14 in an In Vitro Model System of the Gastrointestinal Tract

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Abstract. There is evidence that prebiotics can impart a range of health benefits if consumed on a regular basis. With a growing market for prebiotic-containing foods there is increasing interest in understanding how prebiotics function at the molecular level. Advances in the genomics of lactobacilli and bifidobacteria have enabled modeling of transport and catabolic pathways for prebiotic utilization. The goal of this study was to develop a physiological model of the upper gastrointestinal tract to investigate the metabolic profile of probiotic strain *Lactobacillus bulgaricus* L14 in present of 0.75% polysaccharides obtained from *Plantago major* L. leaves. The polysaccharide degradation and utilization from probiotic strain L14 using *in vitro* GIT system including gastric phase and intestinal phase simulated digestion in humans was developed. As a results of hydrolytic processes in gastric phase after 2h, polysaccharides obtained from *Plantago major* L. leaves were partially hydrolyzed to monosaccharides- mainly galactose and oligosaccharides with degree of polymerization higher than 6. In the conditions simulating intestinal phase, the growth of *Lactobacillus bulgaricus* L14 from $1x10^5$ CFU/ml on the 0h to $4x10^5$ CFU/ml on the 4th hour was observed. D-lactate (15.69 mmol/L) and L-lactate (3.07 mmol/L) were detected after 4h cultivation in conditions simulating intestinal phase. In addition, an *iv vitro* model induced hyperglycemia (100mM glucose) in human erythrocytes, treated 48h with metabolites simulating intestinal phase from 4th hour, showed 41% reduction of catalase and 26% incising of superoxide dismutase activity.

Key words: polysaccharides, *Lactobacillus bulgaricus* L14*, Plantago major, in vitro.*

Introduction

Polysaccharides are type of natural macromolecules usually consist of more than 10 monosaccharides linked through glyosidic bonds in linear or branched chains (Xie et al., 2016). Although plant derived polysaccharides are one of major source of energy, they have a lot of beneficial effects related with reducing the risk factors for some chronic diseases, including diabetes, cardiovascular diseases, hyperlipidemia, certain type of cancer (Lovegrove et al., 2017; Wu et al., 2019), but also have antioxidant, hepatoprotective, anti-inflammatory and immunomodulatory effects (Ren et al., 2017; Zeng et al., 2016; Li et al., 2017).

Plant polysaccharides can evade the action of human digestive enzymes from saliva, stomach and small intestine (Wang et al., 2015; Di et al., 2018), but when they reach large intestine, they can be utilized and broken down by gut microbiota (Di et al., 2018; Chen at al., 2018). As a consequence of this, some health-promoting bacteria's such as *Lactobacillus*, *Bifidobacterium* increased, and production of short-chain fatty acids (SCFAs) in the colon are enhanced (Fernández et al., 2016; Chen at al., 2018). Lukova and colleagues, prove that pectin type water-extractable polysaccharides from *Plantago major* L. leaves and their lower molecular weight hydrolysates

stimulate the growth of four *Lactobacillus* strains: *L. acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27 (Lukova et al., 2020). *P. major* L. leaves have been used in traditional medicine in treatment of a different diseases related to the respiratory organs, skin, digestive organs, against infections and so on. Those effects are owing to variety of biologically active ingredients like polysaccharides, lipids, derivatives of caffeic acid, flavonoids, iridoid glycosides and terpenoids (Samuelsen, 2000).

Studies about food digestion should be performed in vivo but sometimes this is not possible by ethical, technical or financial reasons (Li et al., 2020) Development of *in vitro* digestion methods, static or dynamic, stimulate the complex physiological conditions of the gastrointestinal tract. Static models use a constant ratio of food to enzymes and electrolytes, pH is constant in each digestive phase- oral, gastric and intestinal. Previous studies show that static *in vitro* digestion models could be used for the prediction of the outcomes of *in vivo* digestion (Bohn et al., 2018; Sanchón el al., 2018; Brodkorb el al., 2019). The goal of this study was to develop a physiological model of the upper gastrointestinal tract to investigate the metabolic profile of probiotic strain *Lactobacillus bulgaricus* L14 in present of 0.75% polysaccharides obtained from *Plantago major* L. leaves. To aim this, an *in vitro* model including gastric phase and intestinal phase simulated digestion in humans was performed. In addition, an *in vitro* model induced hyperglycemia on human erythrocytes, treated with metabolites produced in intestinal phase, was developed. Enzymatic activities of catalase and superoxide dismutase was measured in order to verify potential antioxidant activity of polysaccharides obtained from *P. major* L. leaves.

Materials and Methods

Materials and reagent

Acid-extractable polysaccharides from *P. major* L. leaves were obtained by Lukova and colleagues (Lukov et al., 2017). The probiotic strain of *L. bulgaricus* L14 was received from the bacterial culture collection of the department of Biochemistry and Microbiology, Plovdiv University, Bulgaria. Catalase and superoxide dismutase assay kits were purchased from Arbor Assays, Michigan, USA. Hemoglobin assay kit was purchase from Human, Wiesbaden, Germany.

Static in vitro digestion method

Simulated *in vitro* digestion was carried out according to the INFOGEST protocol set up by Minekus et al. (2014)vwith some modification. Gastric and intestinal phase were simulated. For 400mL simulated gastric fluid were needed electrolytes, which were prepared by mixing of 2.76 ml 0.5 M KCl, 0.76 ml 0.5 M KH_2PO_4, 5 ml 1 M $NaHCO_3$, 4.72 ml 2 M NaCl, 0.16 ml 0.15 M $MgCl_2$ x $(H_2O)_6$ and 0.2 ml 0.5 M $(NH_4)_2CO_3$. Three grams of polysaccharides and 0.2 g pepsin were separately dissolved in 50 ml sterilized H_2O. After that, H_2O was added to final volume of 400 ml and pH was adjusted to 3 by 1 M HCl solution. The simulated gastric fluid was incubated for 120 min with shaking (100 r/min) at 37 °C. At 0h and 2h of digestion, 30mL of samples are taken out.

After 2h of digestion, pH was adjusted to 7 by 1 M NaOH solution and in the simulated gastric fluid were added: 2g peptone, 2g yeast extract, 0.168g bail salts and 0.8g trypsin. The probiotic strain of *L. bulgaricus* L14 was cultivated overnight in MRS medium at 37°C. Overnight cells were washed twice in 0.9% NaCl solution and after that were added. The simulated intestinal fluid was incubated for 360 min with shaking (100 r/min) at 37°C. At 0h, 1h, 2h, 3h and 4h of digestion, 30 ml of samples are taken out.

Analytical assays

Bacterial growth

Bacterial growth was measured by a turbidimetric method at 600nm against a cell dry weight standard curve using a UV/Vis spectrophotometer (Beckman Coulter DU 800, USA). Furthermore,

50 µl of taken samples were used to inoculate MRS agar medium (Eddy jet 2W) for period of 24h at 37°C for examination of CFU/ml (Sphere Flash).

After that, samples were centrifuged at 9000 rpm, 20 min, 4 °C and cells were collected. The separated supernatants were used for further analysis of metabolites.

Analysis of metabolites

L-Lactic acid, D-Lactic acid, acetic acid, propionic acid and butyric acid were determined with HPLC system Konik-Tech, with UV Detector (Konik-tech, λ=210nm) and Aminex HPX-87H 5µm (250 x 4,6 mm) column, mobile phase 0.005 M H_2SO_4, flow rate 0.6 ml/min, temperature 40°C. The registered peaks of the samples were evaluated using reference short chain fatty acids standards of: L-Lactic acid, D-Lactic acid, acetic acid, propionic acid and butyric acid.

Analysis of carbohydrates and proteins

The oligosaccharides and residual sugars were analyzed by HPLC system Shimadzu (Japan) coupled with autosampler Nexera X2, SIL-30AC; column over – CTO-20AC and detector RID-20A, Shimadzu (Japan). Ten µl of the sample was injected in the system and eluted into a guard Tracer carbohydrate 5µm 15 x 0.46 column (Tecknohroma, Spain) with mobile phase of 65:35 acetonitrile: water, flow rate 0.78mL/min and temperature 35°C. Results were analyzed with LabSolution, Nexera- XR-RF software using the standard monosaccharides.

Protein content in the samples were measured by the method of Bradford (Bradford, 1976).

Enzymatic activity

The collected bacterial cells were washed twice with 50 mM sodium acetate buffer pH 7.5 and suspended in 2 ml cold lysis buffer which contain 50 mM sodium acetate buffer pH 7.5, 2% glycerol and 30 mM NaCl. The sonication of cells was executed by Tech-pan Ultasonic Disintegrator UD – 20, Warsaw, Poland for 15 cycles and 50% amplitude. Duration of each cycle was 5 sec and 2 min break between them on ice. Thereafter, the lysate cells were centrifuged at 12000 rpm, 10min, 4 °C and supernatants were collected for measure of α-galactoidase, β-galactosidase, α-glucosidase, β-glucosidase and β-xylosidase activity. Alpha-glucosidase activity was determined by the method of Dewi et al. with some modifications, as the amount of p-nitrophenol (pNP) released by the degradation of the pNP-α-D-glucopyranoside substrate (Sigma-Aldrich). The reaction mixture contained 250 µl of 5 mM pNP-α-D-glucopyranoside substrate in 50 mM KH2PO4 at pH 6.8, 150 µl of water, and 100 µl of the bacterial lysate. The mixture was incubated for 10 min at 37 °C. The reaction was stopped by the addition of 2 mL of 1 M Na_2CO_3. The absorbance of the released pNP was measured at 405 nm. Alpha-galactosidase activity of L. plantarum strains was determined by the method of Petek et al. as the amount of pNP released by the degradation of pNP-α-D-galactopyranoside substrate (Sigma-Aldrich). The reaction mixture was composed by 100 µl 9.9 mM PNP-α-D-galactopyranoside substrate in 0.1M KH_2PO_4 buffer and 100 µl of the bacterial lysate. The total volume was brought up to 500 µl with water and incubated for 5 min at 37°C. The reaction was stopped by the addition of 1 mL of borate buffer (pH 9.8). The amount of the released pNP was measured at 405 nm. Beta-xylosidase activity was determined by the method of Lasrado and Gudipati as the amount of pNP released by substrate degradation of pNP-β-D-xylopyranoside (Sigma-Aldrich). The reaction mixture contained 900 µl 5 mM pNP-β-D-xylopyranoside in 50 mM KH2PO4 (pH 5.7), 100 µl water, and 100 µl of the bacterial lysate. The mixture was incubated for 30 min at 30 °C. The reaction was stopped by the addition of 100 µl saturated sodium tetraborate solution. The amount of pNP was measured at 410 nm. 2.4. *In vitro* model induced hyperglycemia.

Erythrocytes were isolated from blood of three healthy young man. First of all, the blood was centrifuged at 3000 rpm, 20 min, 4 °C. Plasma and leukocyte buffy coat were aspirated and the erythrocyte pellet was washed three times with 4 volumes of PBS buffer, pH 7.4. Washed erythrocytes were suspended to a final hematocrit of 50% in PBS containing 5 mM, 50 mM and 100

mM of glucose. To 250 µl of corresponding hematocrit was added 150 µl of sample taken out on 4thh of intestinal phase. The samples were incubated at 37 °C for 24h and 48h with continuous mixing. The samples suspended with 5 mM glucose represented a control group and they simulated the physiological conditions in erythrocytes.

The erythrocytes were lysed by adding ice-cold water in ratio 1:20 (Ferreira et al., 1999). They were centrifuged at 7000 rpm, 10 min. The supernatant was used for measuring activity of catalase and superoxide dismutase and concentration of hemoglobin.

Results and Discussion

As a results of hydrolytic processes in gastric phase after 2h, polysaccharide obtained from *Plantago major* L. leaves were partially hydrolyzed to monosaccharides- mainly galactose (RT- 5.429) and oligosaccharide (RT- 6.666) with degree of polymerization (DP) higher than 6 (Fig. 1A).

After 4h digestion in conditions simulating intestine, hydrolysis of polysaccharide (RT 2.729) and production of monosaccharides continue. The content of monosaccharides probably were: galacturonic acid, galactose and rhamnose. This is proved in study by Lukova and colleagues (Lukova et al., 2017). Oligosaccharides were also cleaved from polysaccharide (RT- 6.788 and RT- 7.029) (Fig. 1B).

In their study, Hu et al. Observed that polysaccharides from seeds of *P. asiatica* L. could be degraded into smaller unites by gastric fluid because of breakdown of glyosidic bonds (Hu et al., 2014).

On the Fig. 2 was presented the growth kinetics of *L. bulgaricus* L14 in terms of OD 600 nm during 4h digestion.

In the conditions simulating intestinal phase in the presence of polysaccharide, the growth of *L. bulgaricus* L14 increased 4 time during process of fermentation - 1x10^5 CFU/ml on the 0h up to 4x10^5 CFU/ml on the 4th hour.

Concentration of SCFAs were presented on Fig. 3. Our study show that D-Lactic acid and L-Lactic acid were primary fermentation products. Their concentrations were increased rapidly after one hour of fermentation and reach the highest values at the 4th hour (D-Lactate 15.59 mmol/L and L-Lactate 3.07 mmol/L). Butyric acid was detected in trace (0.23 mmol/L) at 1st hour. Acetic acid and propionic acid weren't produced by *L. bulgaricus* L14 after 4h of fermentation.

Some plant polysaccharides as a result of fermentation by distinct group of beneficial bacterial species in gastrointestinal tract like *Lactobacillus, Bifidobacterium, Faecalibacterium,* etc. produced SCFAs mainly lactic acid, acetic acid, propionic acid and butyric acid (Fernández et al.,2016). Acetic acid is important energy source for muscles, brain but also is involved in lipogenesis, gluconeogenesis and cholesterol synthesis (Mateos-Aparicio et al., 2016). It also could protect from food allergy via induction of CD103+ DCs and Treg cell response (Ding et al., 2017). Butyrate act as an anti-tumor compound for tumor colonocytes via promoting pro-apoptotic routes (Fernández et al., 2016). SCFAs are associated with reducing risk factors for development of some diseases including the irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease and cancer. SCFAs perform their effects in different cell types by different mechanisms. Binding with specific G protein-coupled receptors (GPR) such as GPR41, GPR43, and GPR109a may mediate SCFAs activities (Offermanns, 2014; Iraporda et al., 2015).

In our study, the activity of β-galactosidase and α-glucosidase were detected during fermentation of polysaccharide. The highest activity of β-galactosidase was measured after 4h of fermentation (0.08 U/mg protein). The same tendency was preserved in the activity of α-glucosidase (0.06 U/mg protein) (Fig. 4). The α-galactosidase, β-glucosidase and β-xylosidase activity weren't detected. This indicate that *L. bulgaricus* L14 strain can produce a enzymes which are able to hydrolyze glyosidic bonds in the plant polysaccharides. The same conclusion but for different *Lactobacillus* strains was proved in study by Lukova and colleagues (Lukova et al., 2020).

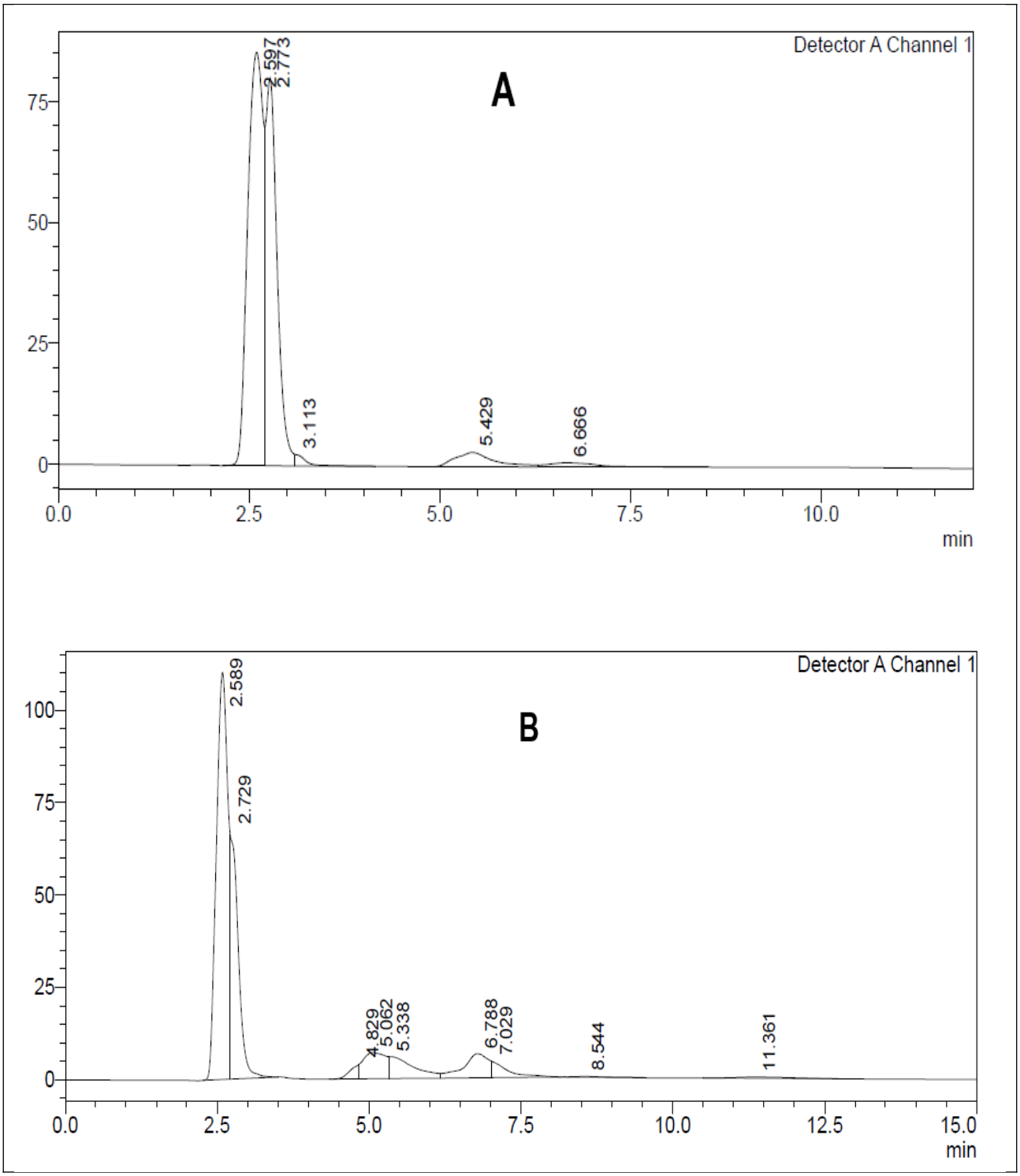

Fig. 1. HPLC profile of polysaccharide hydrolysis products in simulated gastrointestinal tract: (A) gastric phase 2nd hour, (B) intestinal phase 4th hour.

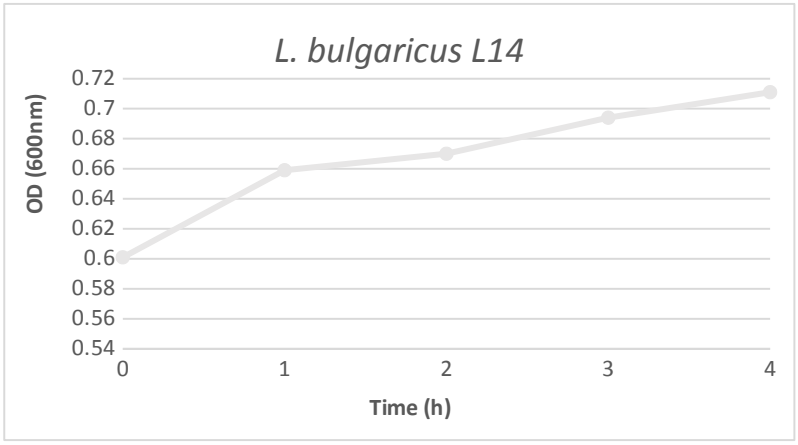

Fig. 2. Optical density value (OD) of *L. bulgaricus* L14 strain in the intestinal phase.

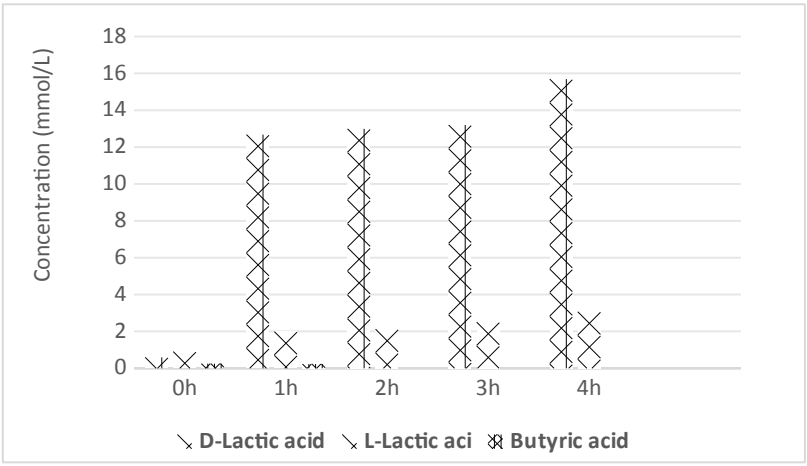

Fig. 3. Concentration of SCFAs at different time points of fermentation in intestinal phase.

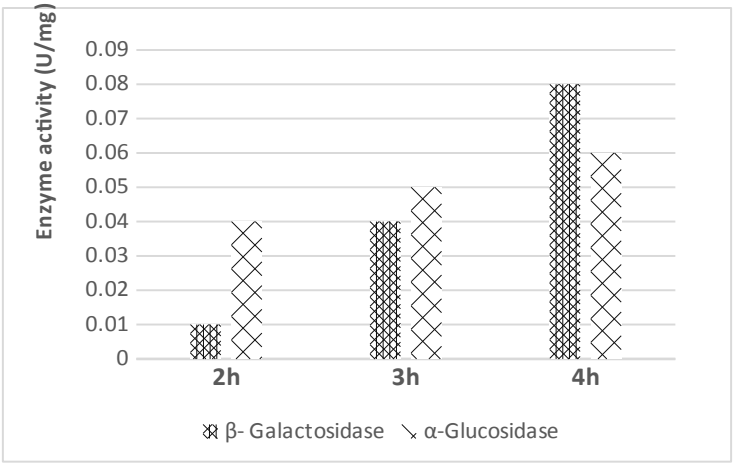

Fig. 4. β-galactosidase and α-glucosidase activity of *L. bulgaricus* L14 strain involved in utilization of polysaccharide.

Lactobacillus and *Bifidobacterium* species are the typical groups of probiotic microbes (Hill et al. 2014). They have genes that encode carbohydrate active enzymes (Flint et al., 2012). *Lactobacillus* species us Embden-Meyerhof pathway and phosphoketolase pathway for utilization of monosaccharides. The depolarization of complex prebiotics by probiotic bacteria involve collaboration between glycosyl hydrolases (GHs) localized intra or extracellular and transport system for hydrolyzed substrates. The genes encoding the transporter components and the related catabolic enzymes for the same substrates are clustered and coregulated as single operons (Goh et al., 2015).

The activity of antioxidant enzymes in protection of erythrocytes from ROS is very important. Our date show that activity of catalase was reduced 41% in erythrocytes treated 48h with metabolites simulating intestinal phase (Fig. 5A). This could be as a result of glycosylation of intracellular proteins caused by high glucose levels.

On the other hand, the activity of superoxide dismutase in experimental groups after treatment 48h in conditions simulating hyperglycemia (50mM glucose and 100 mM glucose) was higher, significantly in the group treated with 100mM glucose, around 26% (Fig. 5B). We can consider that, fermentation product from polysaccharides obtained from *Plantago major* L. leaves activate protective systems in erythrocytes in conditions of high oxidative stress induced by savior hyperglycemia.

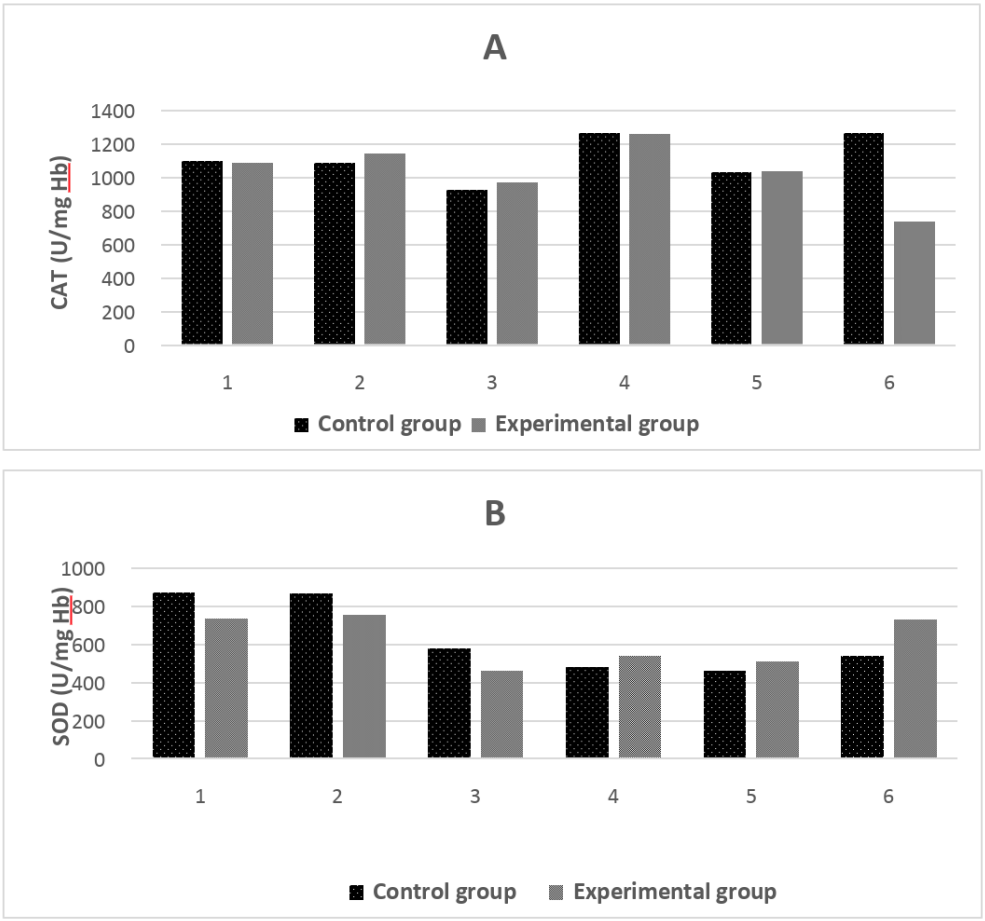

Fig. 5. Catalase and superoxide dismutase activity in erythrocytes treated with metabolites from intestinal phase.

Hyperglycemia is the reason for various changes in different tissues and cells. Erythrocytes as the most abundant cells in human body couldn't escape from those changes. The structure, the functions and the longevity of erythrocytes are impaired. This could be due to high production of reactive oxygen species (ROS) and induction of oxidative stress which lead to lipid peroxidation and disruption of erythrocytes membrane phospholipid bilayer (Pazzini et al., 2015).

Conclusions

The impact of non-digestible plant derived carbohydrates on human microbiota depends on the complex relationship between their chemical composition, bacteria strain specificity and metabolism. The ability of pectin type water-extractable polysaccharides from *Plantago major* L. leaves, as well as their lower molecular weight hydrolysates, to stimulate the growth of probiotic strain *Lactobacillus bulgaricus* L14 strains *in vitro* GIT system was proven. The obtained high amount of lactic acid and the secretion of some glycohydrolases in condition of *in vitro* GIT system are a prerequisite for an in-depth study of the prebiotic capacity of *P. major*. Furthermore, substantial in vivo investigations on the correlation between the probiotic properties of lactobacilli and the prebiotic activity of *P. major* carbohydrates fractions could reveal their potential application as functional food with synbiotic characteristics.

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