ACTA MICROBIOLOGICA BULGARICA

Oligosaccharide Production from Lactose and Lactulose by β-Galactosidase from *Lactobacillus plantarum S30*

Plamena Zheleva, Tonka Vasileva, Veselin Bivolarski, Ilia Iliev*

Department of Biochemistry & Microbiology, Plovdiv University, 24,Tzar Assen Str, 4000, Plovdiv, Bulgaria

Abstract

A β-Galactosidase preparation from ten strains *Lactobacillus plantarum* was evaluated as a biocatalyst for the synthesis of oligosaccharides. Among them, the enzymes from *L. plantarum* S26 *and L. plantarum* S30 were selected for further studies. The correlation between organic acid production, α-galactosidase, β-galactosidase and lactate dehydrogenase activity and the initial concentration of lactose and lactulose was studied.

It could be suggested that the studied β-galactosidase catalyzes the trans-glycosylation reaction at 20% lactose and 20% lactulose concentrations.

Key words: lactose, lactulose, prebiotic potential, *Lactobacillus,* prebiotics, β-galactosidase, α-galactosidase

Резюме

β-Галактозидаза от 10 щама *Lactobacillus plantarum* е изследвана като биокатализатор за синтезата на олигозахариди.Сред тях ензимите от *L. plantarum* S26 *и L. plantarum S30* са селектирани за последващи изследвания. Изследвана е корелацията между продукцията на органични киселини, α-галактозидазна, β-галактозидазна и лактат дехидрогеназна активност от една страна и началната концентрация на лактоза и лактулоза. Получените резултати показват възможностите на изследваната β-галактозидаза да катализира реакция на трансгликозилиране при концентрации на лактоза 20% и лактулоза 20%.

Introduction

Several strains from the genus *Lactobacillus* have been considered as probiotics due to their beneficial effect on the host by improving the intestinal microflora, helping in the immune system maturation, and presenting inhibitory activity toward the growth and adhesion to epithelial cells or intestinal mucus of pathogenic microorganisms (Tannok *et al*., 2004; Mnnoz *et al*., 2012).

Lactobacillus plantarum is a widespread member of the genus *Lactobacillus*, commonly found in many fermented food products, including sauerkraut, pickles, brined olives, Korean kimchi, Nigerian Ogi, sourdough, and other fermented plant materials, and also some cheeses, fermented sausages, and stockfish.

It has also been shown that different non-digestible di- and oligosaccharides as fructo-oligosaccharides (FOS), galacto–oligosaccharides (GalOS), xylo-oligosaccharides (XOS), lactulose, etc. act as prebiotics. Prebiotics are galacto-oligosaccharides that promote the growth of probiotic compounds, and are neither digested nor absorbed in the human gut. Several studies have evaluated the fermentation of prebiotic compounds by LAB strains. GalOS are mixtures consisting of numerous different oligosaccharides varying in their degree of polymerization (DP), structure and glycosidic linkage (Iqbal *et al.,* 2010a). The degree of polymerization of the chain of galacto-oligosaccharides generated by an enzymatic reaction depends on the lactose concentration in the media (Rustom *et al*., 1998). Quantitatively, the amount of the different GOS products present appears to follow the order: di- > tri- > tetra- > higher- saccharides, and the linkages synthesized are predominantly β-(1→6); β-(1→3) and β-(1→2) (Smart, 1993). Trisaccharides, espe-

*Corresponding author: Ilia Iliev e-mail: iliailiev@uni-plovdiv.bg

cially galactosyl 1→6 lactose, can be identified at most lactose levels. Tetra- and higher saccharides have been reported only when using much higher starting lactose levels, although they are considered to be formed at most lactose concentrations but inquantities too small to be detected (Mahoney, 1998).

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is an enzyme that catalyzes two basic reactions, hydrolysis of lactose and structurally related galactosides, and transglycosylation reactions, resulting for example, in a mixture of galacto-oligosaccharides when lactose is the starting material for the latter reaction (Iqbal *et al.,* 2010b). α-Galactosidase enzymes (α-D-galactosil galactohydrolases, E.C. 3.2.1.22) hydrolise the α-1,6 linkages that join the residue of galactose to the glucose present in raffinose, producing free galactose and sucrose. Possible sources of these two enzymes are plant, bacteria, fungi, animal organisms and moulds. Lactic acid bacteria have been studied intensively with respect to their enzymes for various different reasons including their GRAS status. *Lactobacillus plantarum* is a versatile lactic acid bacterium, which is encountered in a range of environmental niches including dairy, meat, and vegetable fermented foods (Saarela *et al.,* 2003).

The aim of the present work was to study the activity α-galactosidase and β-galactosidase from the studied strains *Lactobacillus plantarum* S26 and *Lactobacillus plantarum* S30*,* cultivated in a medium with more than 15% lactose and 15% lactulose as a sole carbon substrate.

Materials and methods

Bacterial strains and culture conditions

In this study two strains, *Lactobacillus plantarum* S26 and *Lactobacillus plantarum* S30, from the collection of the Department of Microbiology, Sofia University, Bulgaria, were used (Stoyanovski et al., 2009). They were identified using 16S RNA techniques. The strains were cultured overnight (16-18 h) on MRS (de Mann Rogosa Sharpe broth, Merck, Darmstadt, Germany) at 37ºC and in limitation of oxygen (BBL® Gas Pak anaerobic system Envelopes, Becton Dickinson, Franklin Lakes, NJ USA).

Carbohydrates used in this study

Lactose (Merck, Germany) and lactulose (Lactulose Crystals EP, Viccio, Italy) contained lactulose 97.5%, galactose 0.5%, lactose 0.5%, epilactose 0.5%, tagatose 0.5%, fructose 0.5%. Glucose (purity 99%, Merck, Germany) was used as a control as well as lactose (Merck, Germany). Each carbohydrate was sterilized on 0.2 μm sterile filter (Sartorius), and pH was not adjusted. All examinations were performed at least twice.

Fermentation

Lactobacilli were routinely grown in MRS broth (Merck). Overnight grown cells were washed twice in saline (0.85% NaCl solution) and 10% of the bacterial suspension (10^7 cfu/mL-1) was used to inoculate mMRS broth medium (pH 6.8) from 5% to 25% lactose and from 2% to 25% lactulose. The anaerobic fermentations were performed for lactobacilli in 50 mL PS bottles at 37ºC for 48 h (Mandadzhieva *et al.,* 2011).

Microbial growth

Bacterial growth was measured by a turbidimetric method at 650 nm and calibrated against cell dry-weight using a spectrophotometer (UV/Vis Beckman Coulter DU 800, USA). For each experiment, data was analyzed using Excel statistical package. The optical density (OD) readings and standard deviations were calculated from duplicate samples from two separate experiments.

Analysis of metabolites

Lactic acid was determined enzymatically with L-lactate dehydrogenase and D-lactate dehydrogenase (commercially available kit code 10 139 084 035, Boehringer, Mannheim, Germany,).

Acetic acid was determined enzymatically with acetyl-CoA synthetase, citrate synthase, malate dehydrogenase (commercially available kit code 10 148 261 035, Boehringer, Mannheim, Germany).

Ethanol was determined enzymatically with alcohol dehydrogenase and aldehyde dehydrogenase (commercially available kit code 10 176 290 035, Boehringer Mannheim, Germany).

Analysis of carbohydrates

Oligosaccharides were analyzed by HPLC using a Symmetry C_{18} column (4.6 x 150 mm) and a Waters 1525 Binary HPLC Pump (Waters, Milford, MA, USA). Oligosaccharides were detected by using a Waters 2414 refractive index detector. The products were identified in the chromatograms as described by Remaud-Simeon *et al.* (1994).

Sugars (residual glucose, xylose, and xylooligosaccharides in fermentation broth after fermentation) were determined by HPLC, using Zorbax carbohydrate column (4.6 x 150 mm; Agilent, Santa Clara, CA, USA), analytical guard column Zorbax NH2 (4.6 x 12.5 mm), and a mobile phase of 75/25 acetonitrile/water. Breeze Chromatography Manager Software (Waters) was used for data treatment.

Enzyme activity

The β-galactosidase activity assays were carried out using ONPG(ortho-Nitrophenyl-β-galactopyranoside) with substrate prepared in citrate-phosphate buffer solution. One β-galactosidase unit (U) was defined as the amount of enzyme which liberated 1 μmol of ONP(ortho-Nitrophenyl) per min per mg of protein at 37°C and pH 6.0 (Kneifel *et al.,* 2000).

The α-galactosidase activity assays were carried out using PNPG (*p*-Nitrophenyl α-D-galactopyranoside) with substrate prepared in citrate–phosphate buffer solution. One α-galactosidase unit (U) was defined as the amount of enzyme which liberated 1 μmol of ONP per min per mg of protein at 37°C and pH 6.5 (Kontula *et al.,* 2002).

Proteins were assayed by the method of Bradford (1976) by using bovine serum albumin as standard (Olano *et al.,* 2009).

Preparation of resting cells

Bacterial cells of *L. plantarum* S26 *and L. plantarum* S30, cultivated in mMRS-10% lactose in the logarithmic phase of growth were harvested by centrifugation at 6000xg for 10 min (4°C), and then washed twice with physiological saline.

Hydrolysis of lactose and lactulose by resting cells

The hydrolysis of lactose and lactulose was performed with 5g-wet/l resting cells in physiological saline at 37°C for 60 min. After boiling for 10 min., the sample was analyzed by HPLC for the detection of galactose, glucose, lactose, lactulose and oligosaccharides as described above.

Results

As a result of the performed screening procedure of lactobacillus strains, it was found that the examined strains utilized 15% of lactose and 5% lactulose with different capacity for changing biomass concentration, pH, and enzyme activity (Zheleva, *et al.,* 2014). The next investigation was focused on the production of organic acids during the cultivation in mMRS media with 15% lactose and 5% lactulose (Tables 1 and 2). The fermentation pattern depends on the physiological conditions of the growing cells. Homofermentative LAB can ferment hexoses via glycolysis with 90% of the glucose being metabolized to lactic acid, and heterofermentative LAB metabolized hexoses to lactic acid, acetic acid and ethanol. It is known that *L. plantarum* is a facultative homofermentative LAB species. When cultivated on 15% lactose and 5% lactulose, the studied strains *L. plantarum* S26 *and L. plantarum* S30 produced different amounts of D-lactate, L-lactate, acetic acid, and ethanol (Tables 1 and 2). When the fermentation end-products, obtained using the high concentration of lactose and lactulose as growth substrates, were compared to those observed on glucose, the main effect was that the production of lactic acid was lower while the production of acetic acid and ethanol increased. Both studied strains showed the same behavior.

In the current study, the activity of lactate dehydrogenase of the two studied strains cultivated on mMRS medium with 5%, 10%, 12,5%, and 15% lactose were determined. The study of the enzyme profiles concluded that the activity of lactate dehydrogenase was not strongly associated with cell growth (data not shown). The activity of the studied enzyme was evaluated during the growth of the strains (Fig. 1). The maximum lactate dehydrogenase activity was found when the strains were cultivated in 5% lactose on the 12th h of the process.

Table 1. Dynamics of organic acids and ethanol producing of *L. plantarum* S26, cultivated on mMRS with 5% lactulose

h	Biomass [g/L]	pH	D-lactate [g/L]	L-lactate [g/L]	acetate [g/L]	ethanol [g/L]
6	1.21±0.9	5.68	0.59±0.1	0.47±0.2	1.84±0.3	0.01±0.001
18	13.8±0.2	3.62	4.71±0.3	5.01±0.1	1.06±0.1	0.02±0.001
24	15.4±0.5	3.34	6.09±0.5	6.16±0.3	1.26±0.3	0.09±0.002
48	15.9±0.8	3.22	6.32±0.1	6.68±0.2	0.94±0.1	0.14±0.3

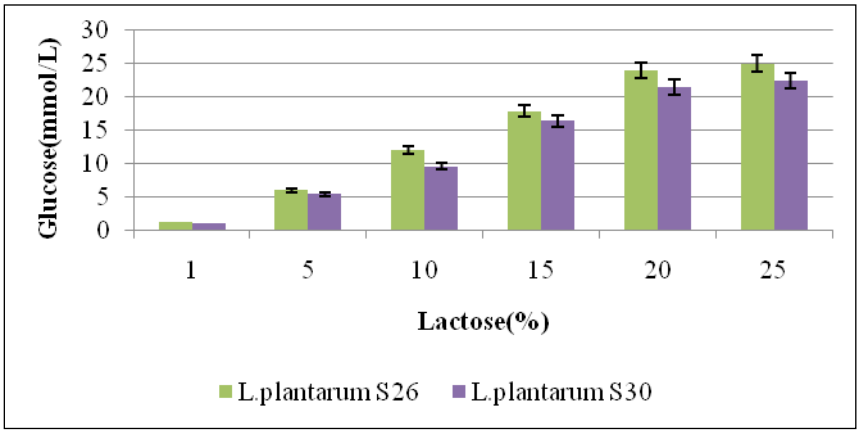
Table 2. Dynamics of organic acids and ethanol producing of *L. plantarum* S30, cultivated on mMRS with 5% lactulose

h	Biomass [g/L]	pH	D-lactate [g/L]	L-lactate [g/L]	acetate [g/L]	ethanol [g/L]
6	0.48±0.1	6.02	0.27±0.1	0.25±0.1	1.89±0.3	0.007±0.0001
18	5.40±0.2	4.27	4.37±0.25	3.99±0.21	1.11±0.2	0.008±0.0003
24	9.90±0.5	3.69	1.99±0.22	4.10±0.28	1.38±0.2	0.001±0.0001
48	12.40±0.8	3.26	6.93±0.25	6.65±0.31	1.40±0.3	0.015±0.009

A 40% (*L. plantarum* S26) and 80% (*L. plantarum* S30) reduction of the enzyme activity was detected when the strains were cultivated in mMRS media with 15% lactose. On the other hand, the maximum lactate dehydrogenase activity was found during cultivation in 12.5% on the 24th h of fermentation for both strains.

Further studies were performed to prove the induction or inhibition of β-galactosidase activity in the presence of lactose and lactulose in concentrations from 1% to 25%. The effect of lactose/lactulose concentration on the hydrolase and transferase activity of β-galactosidase from the studied strains was investigated. Lactose was converted by resting cells of *L. plantarum* S26 and S30 incubated at 37ºC for 24 hours. The total activity was expressed as the concentration of glucose and no inhibitory effect of the concentration of initial lactose for the first hour of the reaction was detected (Fig. 2). Glucose is a non-competitive inhibitor of β-galactosidase as confirmed by the results from 24 h of incubation.

A) Lactose



B) Lactulose

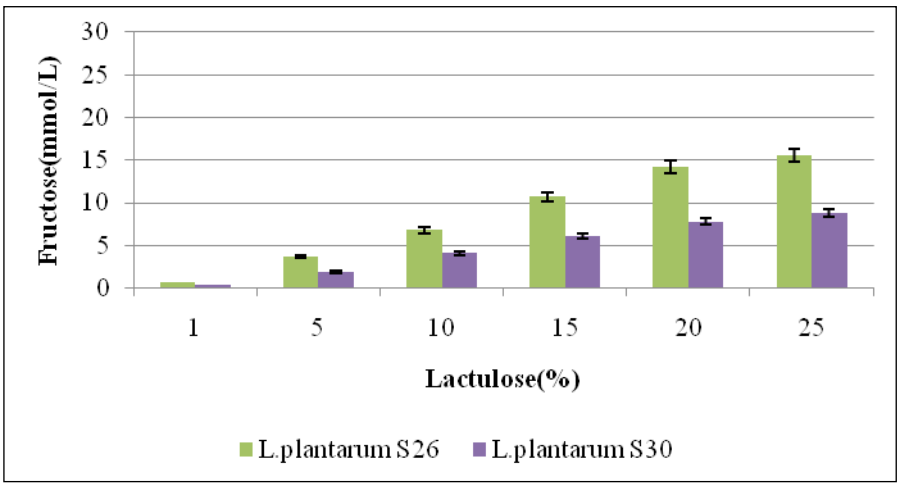
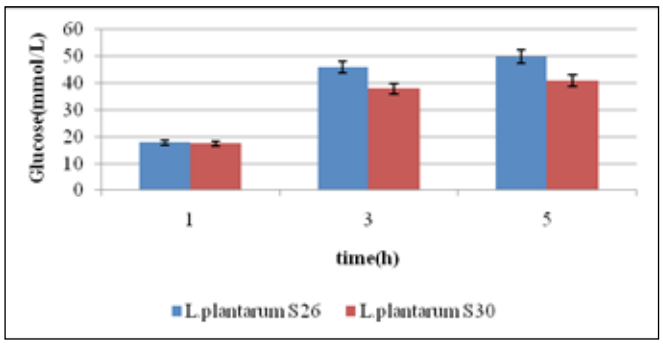
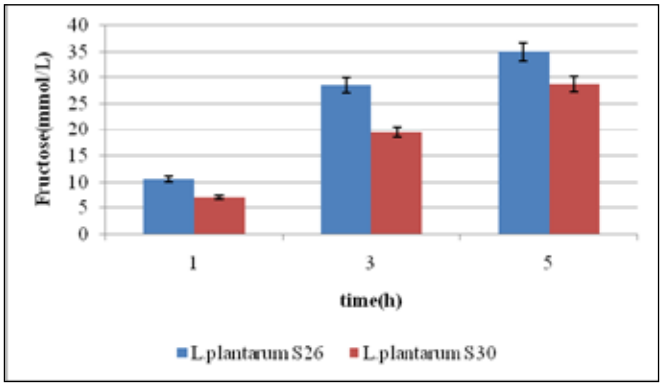


Fig. 1. Hydrolysis on different lactose (A) or lactulose (B) concentrations with β-galactosidases of resting cells of strains *L. plantarum* S26 and *L. plantarum* S30 with duration of the reaction 1h.



A) 15% Lactose



B/ 15% Lactulose

Fig. 2. Hydrolysis of 15% lactose (A) and 15% lactulose (B) with resting cells of strains *L. plantarum* S26 and *L. plantarum* S30

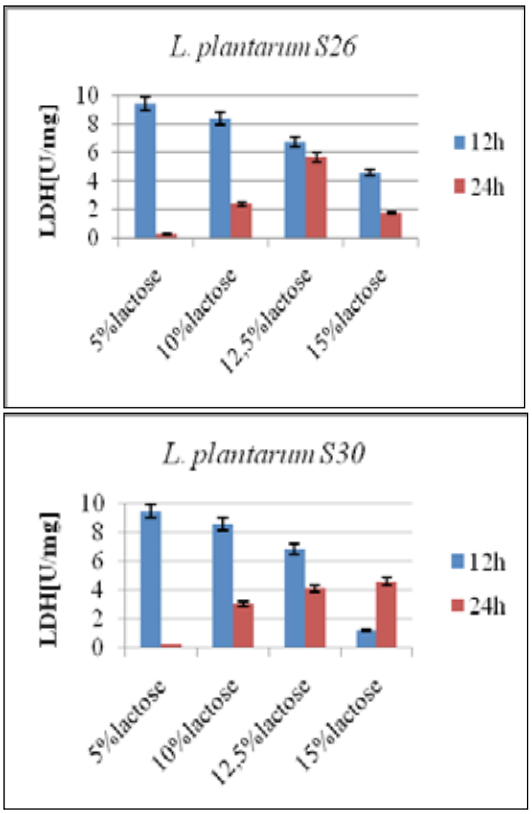


Fig. 3. Dynamics of lactate dehydrogenase activity of strains *L. plantarum* S26 and *L. plantarum* S30, cultivated on media mMRS with 5%, 10%, 12,5% and 15% lactose

According to the results in this study, the formation of galacto-oligosaccharides is limited by the accumulation of glucose during incubation of resting cells for 24h (Fig. 3). When lactulose is a substrate for β-galactosidase from resting cells of *L. plantarum* S26 and S30, the total activity was expressed as the concentration of fructose (Fig. 2).

As shown in Fig. 4. of the transferase reaction, the products synthesized with β-galactosidase from the studied strains displayed two main products, galacto-oligosaccharides with DP 3 and DP 4, using 20% lactose as a substrate (Figure 4). Among the GalOS, only tri- and tetrasaccharides were synthesized and oligosaccharides with longer chains than DP 5 were not found at any time during the course of the reaction. The results demonstrate that the β-galactosidase from strain S26 efficiently synthesized oligosaccharides with DP 3 overall yield of 24% using 25% initial lactose concentration at 40°C. The yield of the GalOS with DP 3 is 14% using 20% initial lactulose concentration.

Discussion

It has been shown in this study that two LAB strains identified as *L. plantarum* S26 and *L. plantarum* S30 isolated from home made sausages can be cultivated in media with lactose in concentration more than 20%. It is well known that most lactobacilli can use 5 % lactose easily, however, this study has shown that only a few strains from *L. plantarum* possess the ability to grow in media with 15% lactose. In this study, it has been demonstrated that in the presence of 12.5% lactose and 5% lactulose, the studied strain *L. plantarum* S26 showed correlation between the highest α-galactosidase and β-galactosidase activities and lactate dehydrogenase activity during the first 12 h of fermentation.

β-Galactosidase (EC.3.2.1.23) catalyzed both reactions – hydrolysis and transgalactosylation, using lactose and lactulose as substrate. In this regard, our results on the enzyme reaction demonstrated *in vitro* the capacity of β-galactosidase from *L. plantarum* S26 to synthesize galacto-oligosaccharides using lactose and lactulose in a concentration from 20 to 25%.

This observation confirms the results described by Smart *et al.* (1993), according to whom high lactose concentrations favor the synthesis of short chain oligosaccharides. It was observed that complete inhibition of oligosaccharide synthesis occurred when the monosaccharide content of the reaction medium reached the total concentration of galacto-oligosaccharides. The optimum reaction

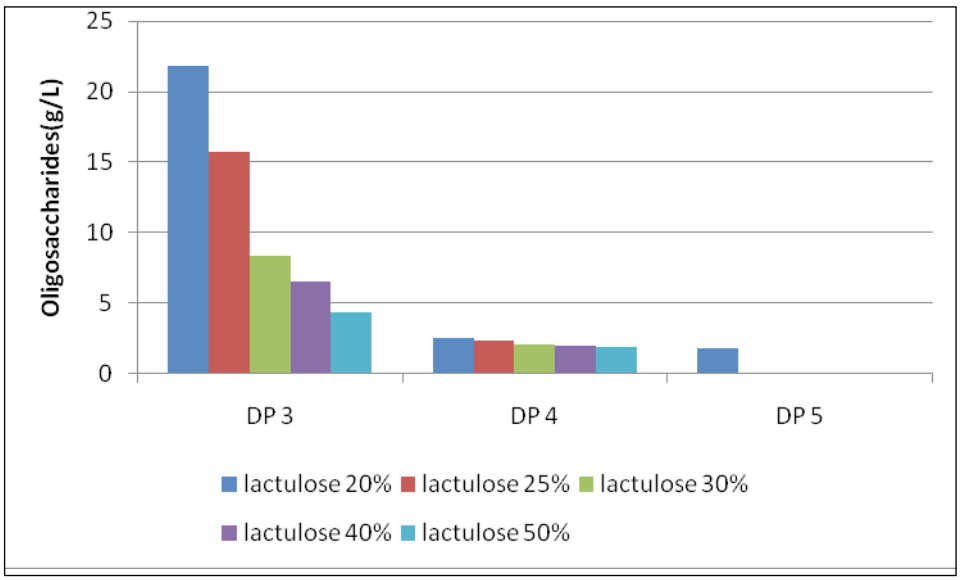


Fig. 4. Distribution of oligosaccharides by degree of polimerisation received through transfer reaction by β-galactosidase of strain *L. plantarum* S26, in the presence of different percentages of lactulose

conditions for galacto-oligosaccharide synthesis with DP 3, substrate concentration and enzyme activity using β-galactosidase from strain *L. plantarum* S26 were determined. Since lactulose is a substrate for β-galactosidase, when the hydrolysis of lactulose is conducted in the presence of fructose, its effect on the hydrolysis of lactulose does not correspond to a competitive inhibitor.

Conclusion

It has been shown in this study that the strain of *L. plantarum* S26 produces α-galactosidase and β-galactosidase during cultivation in a medium with lactose in 15% concentration as a sole carbon source. It could be suggested that the studied β-galactosidase catalyzes the trans-glycosylation reaction at 20% and 25% concentrations of lactose. The selectivity of the reaction of galacto-oligosaccharides synthesis with β-galactosidase was dependent on the enzyme source. The selectivity of the selected enzymes was influenced by the structure and DP of the synthesized oligosaccharides.

Acknowledgements

This work was supported by research grant of NSF Bulgaria BG B01/7-2012.

References

Iqbal, S., T. Nguyen, T., D. Haltrich (2010). β-Galactosidase from *Lactobacillus plantarum* WCFS1: biochemical characterization and formation of prebiotic galactooligosaccharides. *Carbochydr. Res.* **345**: 1408-14016.

Kneifel, W., A. Rajal, K. D. Kulbe (2000). *In vitro* growth behaviour of probiotic bacteria in culture media with carbohydrates of prebiotic importance. *Microb. Ecol. Health Dis.* **12**: 27-34.

Kontula, P., L. Nollet, M. Saarela, T. Vilpponen-Salmela, W. Verstraete, T. Mattila-Sandholm A. von Wright (2002). The effect of lactulose on the survival of *Lactobacillus rhamnosus* in the simulator of the human intestinal microbial ecosystem (SHIME) and *in vivo*. *Microb. Ecol. Health Dis.* **14**: 90–96.

Mahoney, R. (1998). Galactosyl-oligosaccharides formation during lactose hydrolysis: a review. *Food Chem*. **63**: 147-154.

Mandadzhieva, T., T. Ignatova-Ivanova, S. Kambarev, I. I. Ivanova (2011). Utilization of different prebiotics by *Lactobacillus spp.* and *Lactococcus spp*. *Biotechnol. Biotech. Eq.* **25**/2011/4, Suppl: 117-120.

Mnnoz, M., A. Mosquera, C. J. Almeciga-Diaz, O. F. Melendez, A. P. Sanchez (2012). Fructooligasaccharides metabolismand effect on bacteriocin production in *Lactobacillus* strains isolated from ensild corn and molasses. *Anaerobe,* **18**(3): 321-330.

Olano, A., N. Corzo (2009). Lactulose as a food ingredient. *J. Sci. Food Agric.* **89**(12): 1987-1990.

Rustom, I., M. Foda, M. Lopez-Leiva (1998). Formation of oligosaccharides from whey UF-permeate by enzymatic hydrolysis: analysis of factors. *Food Chem*. **62**: 141-147.

Saarela, M., K. Hallamaa, T. Mattila-Sandholm, J. Matto (2003). The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic *Lactobacillus* strains. *Int. Dairy J.* **13**: 291–300.

Iqbal, S., T-H. Nguyen, T. T. Nguyen, T. Maishberger, D. Haltrich (2010). β-Galactosidase from *Lactobacillus planbtarum* WCFS1: biochemical characterization and formation of prebiotic galacto-oligosaccharides. *Carbohydr. Res.* **345:** 1408-1416.

Stoyanovski, S., V. Chipeva, S. G. Dimov, S. Danova, I. Dimitrova, L. Yocheva, S. Atanasova-Nikolova, I. Ivanova (2009). Characterization of lactic acid bacteriafrom dry sausages. *Biotechnol. Biotech. Eq.* **23**/2009/Se *Special Edition/on-line,* 598-601.

Tannok, G.W. (2004). A special fondness for lactobacilli, *Appl. Environ. Microbiol*. **70**: 3189-3194.

Smart, J., C. Pillidge, J. Garman (1993). Growth of lactic acid bacteria and bifidobacteria on lactose and lactose-related mono-, di-, and trisaccharides and correlation with distribution of β-galactosidase and phospho-β-galactosidase. *J. Diary Res.* **60**: 557-568.

Zheleva, Pl., T. Vasileva, T. Mandadjieva, I. Ivanova, I. Iliev (2014). Influence of lactose concentration on the α-galactosidase and β-galactosidase activity of *Lactobacillus plantarum*, *J. BioSci. Biotech. Se/online*: 71-74.