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POTENTIAL OF SOYA SUBSTRATES FOR LACTIC ACID FERMENTATION WITH ADDITION OF *Lactobacillus plantarum*

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POTENTIAL OF SOYA SUBSTRATES FOR LACTIC ACID FERMENTATION WITH ADDITION OF *Lactobacillus plantarum*

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Abstract: In this work, growth dynamics of Fresco DVS 1010 culture and human derived isolate *Lactobacillus plantarum* in milk- water- or lactose-free milk-based soya substrates with sucrose or flavouring compounds (chocolate or caramel) were evaluated. Static anaerobic fermentation was carried out for 8 hours at 37 ± 0.5°C (5% CO_2), afterward with storage period for 3 weeks at 6 ± 0.5°C. Although milk is a typical growth medium for lactic acid bacteria, presumable viable counts of Fresco culture reached levels 10^9 CFU mL^{-1} after 8 h. Potentially probiotic isolate *L. plantarum* added after the fermentation process maintained its viability during cold storage above the limit 10^6 CFU mL^{-1}.
Furthermore, the effect of temperature (8-40°C) on the growth dynamics of *L. plantarum* in a model environment of UHT lactose-free milk (1.5% fat) was investigated. Final densities of studied isolate reached counts 10^7 CFU mL^{-1} in stationary phase at all studied temperatures (except for marginal 8°C). During the growth and multiplication of *L. plantarum*, as a result of metabolic activity, the decrease of pH from initial values about 0.17-1.16 units was recorded. Experimentally, it was found that optimal temperature was close to 34°C, where the fastest growth rate in an exponential phase was recorded (Gr = 0.1684 log CFU mL^{-1} h^{-1}, t_d = 1.8 h).

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POGODNOST SOJINIH SUPSTRATA ZA MLIJEČNO KISELU FERMENTACIJU UZ DODATAK BAKTERIJE *Lactobacillus plantarum*

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Sažetak: U ovom radu ocijenjena je dinamika rasta Fresco DVS 1010 kulture i humanog izolata *Lactobacillus plantarum* u sojinim supstratima na bazi mlijeka, vode ili bezlaktoznog mlijeka s dodatkom saharoze ili okusa (čokolada ili karamela). Statička anaerobna fermentacija provedena je tijekom 8 sati na 37 ± 0,5°C (5% CO_2), nakon čega su proizvodi uskladišteni na 3 tjedna pri 6 ± 0,5°C. Iako je mlijeko tipičan medij za rast bakterija mliječne kiseline, nakon 8 h broj Fresco culture dosegao je brojku od 10^9 CFU mL^{-1}. Broj potencijalno probiotičkog izolata bakterije *L. plantarum* dodanog nakon fermentacije ostao je iznad granice od 10^6 CFU mL^{-1} tijekom hladnog skladištenja.
Nadalje, istražen je učinak temperature (8-40°C) na dinamiku rasta bakterija *L. plantarum* u modelu okoliša UHT bezlaktoznog mlijeka (1,5% masti). Konačna gustoća promatranog izolata dosegla je brojke 10^7 CFU mL^{-1} u stacionarnoj fazi pri svim promatranim temperaturama (osim marginalne 8°C). Tijekom rasta i razmnožavanja bakterija *L. plantarum* zabilježen je pad pH od početnih vrijednosti oko 0,17-1,16 jedinica kao rezultat metaboličke aktivnosti. Eksperimentalno je utvrđeno kako je optimalna temperatura za rast blizu 34°C, gdje je zabilježena najbrža stopa rasta u eksponencijalnoj fazi (Gr = 0,1684 log CFU mL^{-1} h^{-1}, t_d = 1,8 h).

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TABLE OF CONTENT

1. INTRODUCTION

During the past two decades, the food industry is directing the development of new products towards the area of functional foods due to increasing consumer's awareness regarding health with improving quality of life. Beyond the basic nutritional functions, functional foods are expected to have potential benefits to promote health and, therefore, more attention is paid to them. Recently, research in the area of functional foods has moved progressively towards the development of dietary supplementation, introducing the concept of probiotics and prebiotics that may affect gut microbial composition (Bahadoran and Mirmiran, 2015; Charalampopoulos et al., 2002). Currently, industrial foods containing probiotics are most frequently incorporated in dairy products such as yoghurts or fermented milk. Due to the increased number of consumers that have to restrict the dairy consumption (due to lactose intolerance, cow's milk allergy, low-protein diet, phenylketonuria) (Němečková et al., 2011), the development of non-dairy probiotic products could variegate the offer of probiotic foods on the market. There is also considerable increasing potential in manufacturing fermented functional foods for a specific group of consumers based on cereals, pseudocereals or legumes (Matejčeková et al., 2017). Legumes such as soya may be introduced as a functional food representing a rich source of bioactive peptides, dietary fibers and phytochemicals. Moreover, soya is the most common replacement for meat in a vegetarian diet. Therefore, in this thesis, the practical application of potential probiotic isolate *Lactobacillus plantarum* and mixed Fresco culture in real products based on soya will be evaluated. More precisely, the characteristics of growth of selected starter culture (Fresco DVS 1010) and *L. plantarum* will be observed during 21 days, as that is expected to be the shelf-life of the final products. All of that will be assessed by using the tools of predictive microbiology. Water version of substrates will be prepared for people who avoid milk while for lactose intolerant people, lactose-free milk substrates will be made.

Although the technologically important parameters like optimal pH and temperature for industrially used strains are well known, the alterations in the quantitative growth characteristics like growth rate with changing environmental factors are relatively poorly studied. As temperature represents an important factor in the microorganism growth, in control of bioprocesses and safe handling of goods, especially in the food industry, describing the temperature effect on the microbial growth parameters is required. That is why one part of this thesis deals with the quantification of temperature effect on the growth of human derived potential probiotic isolate *L. plantarum* in a model growth media. Since numerous studies on various species of lactobacilli in milk were already conducted, this one gives the knowledge of

the behaviour of *L. plantarum* in lactose-free milk (1.5% fat). This data will help to optimise the processing conditions in case the probiotic isolate will be used as a part of a starter culture in dairy products or as a dietetic supplement.

2. THEORETICAL PART

2.1. SOYA

The soybean plant (*Glycine max*), as one of the most important crops worldwide, belongs to the legume family with world annual production reaching more than 300 million tonnes. It is a traditional oriental food, originated in North and central China and its cultivation started approximately 5000 years ago (Liu, 2016). The soybean, then known as *shu*, was considered as one of the five sacred grains, along with rice, wheat, barley and millet. As one of the most important sources of dietary proteins and oil, it has been called "yellow jewel," "great treasure," "nature's miracle protein," and "meat of the field" (Liu, 1997). Because of its unique chemical composition, the soybean represents one of the most economical and valuable agricultural commodities. In comparison to cereals and other legume species, it has the highest protein content (about 40%). The soybean also contains about 20% oil, the second highest content among all food legumes (after the peanut). The remaining dry matter is composed of mainly carbohydrates (about 35%) and ash (about 5%). Other minor components found in soybeans include minerals, vitamins and various biologically active substances (Liu, 1997).

2.1.1. Soy protein

Soybeans are one of the few vegetarian sources of total proteins containing all of the essential amino acids needed to fulfil human nutritional requirements for growth, maintenance and physical stress (Michelfelder, 2009). Although now soy protein is being recognized as complete protein, previously was considered to be deficient in methionine. It was so because protein-efficiency ratio was the standard method of evaluating protein quality (Messina, 1999). This method was based on the growth of laboratory animals (most commonly rats) but it was proven that rats have 50% higher methionine requirements than humans (Sarwar et al., 1989). The World Health Organization (WHO) and the US Food and Drug Administration have adopted an alternative method for evaluating protein quality called the protein digestibility corrected amino acid score (PDCAAS) (FDA, USDHHS, 1991). Based on this method, soy protein is considered to have a similar equivalent in protein quality to animal proteins (egg white has a score of 1.00, soy concentrate 0.99, beef 0.92, and isolated soy protein 0.92) (FAO/WHO, 1991). Soy protein assures all amino acids in the amount needed by an adult at a dose of at least 0.6 g/kg BM (Young, 1991).

Based on the protein concentration, products containing soy protein may be divided into major groups, whereas whole soybeans contain about 40% protein, soy flour, soy-protein concentrate and isolated soy protein contain 50%, 70% and 90% protein, respectively. Soy flour

is grounded from high-quality, cleaned, dehulled soybeans after most of the oil has been removed. Soy-protein concentrate is a further refined product prepared from high-quality, cleaned, dehulled soybeans by removing most of the oil and water-soluble nonprotein constituents, whereas isolated soy protein is the major proteinaceous fraction prepared from high-quality, clean dehulled soybeans by removing most nonprotein components (Waggle and Kolar, 1979).

Soy proteins are often used to enhance the nutritional quality of other vegetable proteins. Amino acids that are limited in other proteins are present in excess amounts in a soy protein products. For example, soy protein products contain a level of lysine which exceeds human requirements. Hence, supplementation with soy protein products provides an excellent way to correct the lysine deficiency in some grains, such as wheat or corn (Endres, 2001).

2.1.2. Carbohydrates

In comparison to other legumes, soybeans contain lower amounts of carbohydrates, but despite of this, they represent the second largest component. However, the economical value of soy carbohydrates is considered as less important than soy protein and oil. Of the total carbohydrates, 37% is represented by starch, 41% sugars and 22% oligosaccharides. The most common sugars are sucrose, fructose and glucose (Fehily, 2016).

A limited use of soybeans in human diet is due to the flatulence caused by soluble carbohydrates such as raffinose and stachyose. Humans lack the enzymes to hydrolyze the galactosidic linkages of raffinose and stachyose to simpler sugars (Perkins, 1995). However, due to poor digestion by intestinal enzymes, soybean oligosaccharides are classified as prebiotics because in the colon are able to stimulate the growth of positive bacteria, such as bifidobacteria (Messina, 2016).

2.1.3. Fats

Soya is higher in fat in comparison to other legumes that are generally almost fat-free. Soybeans contain about 16% of saturated fatty acids, 24% monounsaturated and 60% polyunsaturated fatty acids. The polyunsaturated fatty acids present in soybean are mostly essential: linoleic and alpha-linolenic acid. All legumes, including soy, have no cholesterol (Fehily, 2016).

2.1.4. Minerals and vitamins

Among major minerals in soybeans, pottasium is found in the highest concentrations followed by phosphorus, magnesium, sulfur, calcium, chloride and sodium. The minor minerals present in soybeans are iron, zinc, manganese, copper, selenium and iodine. Soybeans also

contain both water-soluble and oil-soluble vitamins. The water-soluble vitamins present in soybeans include thiamin, riboflavin, niacin, pantothenic acid and folic acid. The oil-soluble vitamins present in soybeans are vitamins A and E (Liu, 2016).

2.1.5. Bioactive compounds

A large number of heat-stable and heat-labile substances are naturally found in soybeans. These compounds may have adverse or beneficial effect to humans. Therefore, some substances are known as antinutritional factors, while others are referred to as phytochemicals or nutraceuticals (Liu, 2016).

Raw soybeans contain certain proteins (protease inhibitors) that are able to react with digestive enzymes, thereby interfering with the digestion of proteins and starch. There are two types: Kunitz trypsin inhibitors and Bowman-Birk inhibitors. In humans, raw soy or isolated protease inhibitors increase levels of cholecystokinin and pancreatic secretion that may lead to pancreatic hypertrophy, hyperplasia and possibly to cancer. However, protease inhibitors are inactivated by heat and possess no problem in cooked beans (Fehily, 2016).

Other antinutritional factors naturally present in raw soybeans and other legumes are lecitins, also known as hemagglutinins because of agglutination of red blood cells. They are heat labile and inactivated when the beans are properly cooked. If not, they may cause nausea, vomiting, diarrhoea and abdominal pain (Fehily, 2016).

Phytic acid, also known as inositol hexakisphosphate (IP6), represents the main form of storage of phosphorus and inositol in seeds of cereals, legumes and oilseed. It contributes to the poor mineral bioavailability of beans as humans don't have sufficient activity of phytases that are capable of releasing the phosphate group from phytic acid. However, during processing, storage, fermentation, germination and digestion of grains and seeds IP6 may be partly dephosphorylated to produce compounds that have low capacity to bind to the minerals. Furthermore, these compounds may have antioxidant properties (Cabrera-Orozco et al., 2013; Martino et al., 2011).

Isoflavones represent a class of phytoestrogens, plant-derived compounds with estrogenic activity. Soybeans and soy products are the richest sources of isoflavones in the human diet (up to 3 mg/g dry weight). Digestion or fermentation of soybeans results in the release of the sugar molecule from the isoflavone glycoside, leaving an isoflavone aglycone. Soy isoflavone glycosides include genistin, daidzin and glycitin, while the aglycones are called genistein, daidzein and glycitein. Among all the health-promoting components, isoflavones are

thought to be the most responsible for many of the hypothesized health benefits of soyfoods (Liu, 2016; Higdon, 2004).

Soya saponins were originally considered to be toxic because of the structural analogy with saponins from the other sources that are presented as toxic. However, soya saponins have been shown to exert no toxic effects, and its hypocholsterolemic and anticancer properties have been reported. On the other hand, during soaking and blanching, portions of saponins are dissolved in water and lost because of its natural thermal sensitivity (Lee et al., 2005; Shi et al., 2004).

Phytosterols are lipid-like compounds found in plants. Although structurally similar to cholesterol found in humans, they have been clinically proven to reduce blood cholesterol in human bodies. Soybeans, rapeseeds and coniferous trees represent three major commercial sources of phytosterols (Liu, 2016).

2.1.6. Health benefits of soya

One of the health benefits often mentioned is lowering effect of soy protein on LDL-cholesterol that is a well-established risk factor for coronary heart diseases. This benefit was first formally recognized in 1999 by American Food and Drug Administration establishing health claim that "Intake of 25 grams of soya protein a day as a part of a diet low in saturated fat, may reduce the risk of heart disease" (FDA, USDHHS, 1999). The FDA requires for the claim that a serving contains at least 6.25 g of soy protein. Nevertheless, there exists some controversy about the hypocholesterolemic effect of soy protein. American Heart Association (AHA) concluded in an advisory that intake of a very large amount of soy protein, more than half of the daily protein intake, may lower LDL-cholesterol by a few percentage points (Sacks et al., 2006). However, a recent review suggests that soy protein directly lowers circulating LDL-cholesterol and may also modestly lower blood pressure. The replacement of commonly-consumed sources of protein in Western diets by soyfoods may also lead to a favorable change in the fatty acid content of the diet (Messina, 2016).

Soy consumption is also associated with decreasing incidence of many cancers, including breast cancer. Although *in vitro* and animal studies have produced conflicting results, epidemiologic studies have reported a reduced risk of breast cancer in Asian countries where soy consumption is much higher than in Western population (Wu et al., 2015). Also, Zhang et al. (2017) suggest that breast cancer risk is also reduced in North American women consuming soy products regularly. Nevertheless, the estrogenic effect of soy isoflavones has raised concern for a potential increased risk of breast cancer in Western countries, where usual soy

consumption is low (Kucuk, 2017). On the other hand, the European Food Safety Authority concluded that isoflavone supplements do not increase breast cancer risk when taken by postmenopausal women (EFSA, 2015). The American Cancer Society and the American Institute for Cancer Research (AICR) have concluded that soyfoods can be safely consumed by breast cancer patients (Rock et al., 2012; AICR, 2012).

Clinical research which has indicated that soy isoflavones reduce bone loss is mixed. Initial speculation that soyfoods might promote bone health in postmenopausal women was based on the estrogen-like effects of isoflavones and early research showing that the synthetic isoflavone, ipriflavone, exerted skeletal benefits (Brandi and Gennari, 1993). Three meta-analyses concluded that isoflavones favorably affect bone turnover and/or bone mineral density (BMD) in postmenopausal women (Taku et al., 2010; Ma et al., 2008a; Ma et al., 2008b). On the other hand, of the four long-term clinical trials, only one trial found that isoflavones significantly improved BMD (Tai et al., 2012; Levis et al., 2011; Alekel et al., 2010; Marini et al., 2008). Recently published clinical study suggests that more moderate doses of isoflavones (100 mg/day) may prove to be more efficient for promoting bone health than pharmacologic doses (Pawlowski et al., 2015).

During menopause, the level of estrogen declines, that may be the reason why therapies using phytoestrogens, including isoflavones found in soybean, appear to be effective against menopausal symptoms (Messina, 2016). It was found that soybean isoflavones significantly reduced the frequency and severity of hot flashes by 20.6% and 26.2%, respectively. Also, it was reported that supplements containing more than 18.8 mg genistein lead to the reduction in hot flashes frequency more than twice in comparison to those with lesser amounts of genistein. Approximately 40 mg of total isoflavones derived from whole soybeans provides the amount of genistein shown to be more efficiently (Taku et al., 2012).

Soyfoods should definitely be incorporated into the diet as a part of an overall healthy diet and by displacing less healthy foods. The recent update of the Dietary guidelines for Americans recommended an increasing soy intake as fortified beverages and other soy products. The recommended intake is 5 ounces per week, an amount based on 2,000-calorie intake (USDA, 2015). Over the centuries, the Chinese gradually developed many nutritious foods out of soybeans, including soy milk, tofu, soy sprouts, fermented soy paste and soy sauce. Today, soyfoods come in various types: traditional, modern (Westernized), fermented and nonfermented.

2.1.7. Fermentation of soya

Fermentation is one of the oldest and the most economical methods of preservation foods. The preparation of fermented foods has a long tradition both in Southeast Asia and Africa, while today the fermented food industry is one of the largest worldwide. Well known fermented products range from alcoholic beverages such as beer and wine to cheese, soured milk products, various types of bread, yeast products and antibiotics. Fermentation utilizes microorganisms for the transformation of raw materials (substrates) into useful metabolites (Deshpande et al., 2000). The microorganisms used for fermentation include mostly bacteria, yeasts and filamentous fungi (molds). Among them, lactic acid bacteria (LAB) are the most commonly used microorganisms, known also as starter cultures (Mani and Ming, 2016; Marko et al., 2014).

Soybean cannot be eaten raw, thus, it is usually processed to produce a variety of food products. The most common process of preparation is fermentation. Different countries in Asia use different terms for fermented soybean food products that are based on preparation and the type of microbial strain used. Most commonly fermented soybean products are natto, miso (Japan), tempeh (Indonesia), cheonggukjang (Korea), soy sauce and douchi (China). Due to its health and nutritional aspects, traditional fermented soybean products may be considered as functional foods to help in the prevention of various diseases and disorders (Mani and Ming, 2016).

Fermentation of soybean is important in terms of prolonging the shelf life of final products, as well as in improving nutritional properties. Several studies have reported the increasing level of vitamins, such as B_{12}, B_9, E and K (Ginting and Arcot, 2004; Astuti et al., 2000; Morishita et al., 1999; Liem et al., 1977). Furthermore, during the fermentation process, pH decreases, that provides optimum pH values for the enzymatic degradation of antinutritional factors such as phytate. This may lead to an increasing solubility of iron, zinc and calcium and its bioavailability (Emire and Buta, 2015; Marko et al., 2014). Compared to unfermented soybean, fermented soybean foods contain more aglycones than the predominant isoflavone structures. The conversion of glucosides into its aglycone form is efficiently done by fermentation leading to an increased antioxidative activity (Donkor and Shah, 2008; Chien et al., 2006; Pyo et al., 2005).

Fermentation process enhances the appearance, flavour and aroma of products that lead to an improvement of acceptability of soybean products (Mital and Steinkraus, 1974). Undesirable beany flavours contained in soybeans are usually destroyed during the process itself (Desphande et al., 2000). One of the very important features of fermented foods is often

easier digestibility than unfermented foods. During the fermentation process, the enzymes produced by the presented mycrobiota reduce the content of soy oligosaccharides, stachyose and raffinose, often causing digestive problems (Cruz et al., 1981; Mital and Steinkraus, 1974).

2.2. LACTIC ACID BACTERIA

At the beginning of the 20th century, the term "lactic acid bacteria" (LAB) was used to refer to "milk-souring organisms". Traditionally, LAB have been associated with food and feed fermentations and are generally considered as beneficial microorganisms with some strains even as health-promoting (probiotic) bacteria. The monograph by Orla - Jensen (1919) formed the basis of the present classification of LAB that take into account the cellular morphology, mode of glucose fermentation, temperature ranges of growth and sugar utilization possibilities. Taxonomically, LAB are divided into two distinct phyla: *Firmicutes* and *Actinobacteria*. Within the *Firmicutes* phylum, genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Carnobacterium*, *Weissella*, *Alloiococcus*, *Symbiobacterium* and *Vagococcus* belong. Within the *Actinobacteria* phylum, LAB belong to the *Atopobium* and *Bifidobacterium* genera (Liptáková et al., 2017; von Wright and Axelsson, 2012).

LAB form heterogeneous group of gram positive, non-motile, non-sporulating rods and cocci devoid of cytochromes that are catalase- and oxidase- negative, acid tolerant microorganisms, most of which are non-respiring but aerotolerant anaerobes. They produce lactic acid as one of the main fermentation products of carbohydrates. LAB tend to be nutritionally fastidious, often requiring specific amino acids, B-vitamins and other growth factors (Liptáková et al., 2017; Wedajo, 2015).

2.2.1. Metabolism of LAB

LAB may be divided into three groups according to the utilization of sugars: homofermentative, heterofermentative and facultatively heterofermentative. Homofermentative LAB such as *Pediococcus*, *Streptococcus*, *Lactococcus* and some lactobacilli produce lactic acid as the major or sole end-product of glucose fermentation. They use the Embden-Meyerhof-Parnas pathway to generate two moles of lactate per mole of glucose. Heterofermentative LAB such as *Weissella*, *Leuconostoc* and some lactobacilli strains produce equimolar amounts of lactate, CO_2 and ethanol from glucose via the Warburg-Dickens (pentose phosphate) pathway. Facultatively heterofermentative lactobacilli are able to metabolize hexoses via both pathways, but Warburg-Dickens pathway is predominant in case

of lack of fermentable sugars (Kocková et al., 2011; Rattanachaikunsopon and Phumkhachorn, 2010).

Selected strains of LAB are also able to ferment hexoses other than glucose (mannose, galactose, fructose). They enter the major pathways as glucose-6-phosphate or fructose-6-phosphate after the process of isomerisation and phosphorylation. Pentoses can only be fermented by heterofermentative LAB entering the pathway as either ribulose-5-phosphate or xylulose-5-phosphate. As a consequence the CO_2 is not produced (von Wright and Axelsson, 2012). Some LAB metabolize disaccharides such as cellobiose, lactose, maltose, melibiose, sucrose, etc. These sugars are transported across the cell membrane either as free sugars or phosphorylated and are then split into two monosaccharides or a monosaccharide and monosaccharide phosphate (Axelsson, 1993).

Fermentation of organic acids also plays an important role in the metabolism of LAB. Most of the LAB, with few exceptions, are able to convert malate and catabolise citrate to lactate. Amino acids are also potential substrates for pyruvate and lactate formation (Liu, 2003). As free amino acids are scarce in milk, some dairy LAB have proteolytic activities to obtain amino acids from milk casein. The proteolytic system involved in casein utilization provides cells with essential amino acids during growth in milk and is also of industrial importance due to its contribution to the development of the organoleptic properties of fermented milk products (Savijoki et al., 2006). Several dairy LAB, including *Lactobacillus casei*, *L. plantarum* and *L. rhamnosus* have been reported to have lipolytic activity that is important in the development of flavour in dairy products, especially in cheese ripening (Endo and Dicks, 2014).

2.2.2. Probiotics

Strains mainly of lactic acid bacteria used for fermentations having beneficial effect on host health by improving intestinal microbial balance are called probiotics. This term is derived from the Greek “probios” which means “for life”. According to the Food and Agriculture Organization and the World Health Organization (FAO/WHO, 2001) probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. To have a beneficial effect on the host, probiotic microorganisms need to be viable, active and abundant in the concentration of at least 10^6 CFU/mL or CFU/g in the product throughout the specified shelf life. Minimum therapeutic daily dose is usually considered as 10^8 to 10^9 CFU/ml or g (Liptáková et al., 2017; Østlie et al., 2003). The most common types of microorganisms used as probiotics are predominately lactic acid bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* (Soccol et al., 2010; Marco et al., 2006; Shortt,

1999). The most often mean of administration is a fermented dairy product (Ouwehand et al., 2002).

There are some criteria for the selection and assessment of probiotic microorganisms. The characteristics that must be fulfilled are: human origin, nonpathogenic behavior, resistance to technological processes, acid and bile tolerance, ability to adhere gut epithelial tissue and colonize the intestinal tract, antimicrobial activity against intestinal pathogens, modulation of the immune response and the ability to influence metabolic activities (Bielecka, 2006; Mishra and Prasad, 2005).

Functional properties of probiotics have been demonstrated for various therapeutic applications: diarrheal diseases, inflammatory bowel disease, irritable bowel syndrome, prevention of colon cancer, improvement in lactose metabolism, reduction in serum cholesterol, immune system stimulation, suppression of *Heliobacter pylori* infection (Florou-Paneri et al., 2013; Shah, 2007). It is important to note that health benefits provided by probiotics are not species- or genus-, but strain-specific. Therefore, no probiotic strain will provide all proposed benefits (Figueroa-Gonzalez et al., 2011; Shah, 2007).

2.2.3. Genus *Lactococcus*

The genus *Lactococcus* comprises of seven species: *Lactococcus lactis* (including the subspecies *Lactococcus lactis* subsp. *cremoris*, *lactis* and *hordinae*), *L. garvieae*, *L. piscium*, *L. plantarum*, *L. raffinolactis, L. chungangensis* and *L. fujiensis.* Bacteria involved in this genus are mesophilic and are able to ferment hexoses homofermentatively producing L(+) lactic acid requiring complex nutrients (von Wright, 2012). Cells of *Lactococcus* genus are spherical or ovoid in shape, occurring singly, in pairs or in chains (Ward et al., 2002). Optimum growth temperature is 30°C but they are able to grow at temperatures as low as 10°C but not at 45°C (Batt, 2014).

Strains belonging to the species *Lactococcus lactis* are used for acid production in dairy fermentations and represent the most important organisms in the manufacture of wide range of fermented dairy products such as sour milk, cream, butter, fresh cheese and many varieties of semi-hard cheese (Samaržija et al., 2001). The *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* may be differentiated on the basis of growth temperature (subsp. *lactis* strains are generally able to grow at higher temperatures), salt tolerance (*lactis* (4%); *cremoris* (2%)) and ability to utilize arginine (subsp. *cremoris* strains cannot). As cheese starters, *L. lactis* subsp. *cremoris* have less diverse biochemical activities than subsp. *lactis* and are less likely to cause bitterness and other flavouring defects in cheese (Ward et al., 2002).

Lactococci are employed either as single strain starters or as a part of a multiple-strain starter mix, frequently in a combination with other lactic acid bacteria, including *Lactobacillus* and *Streptococcus* strains. Starter cultures impart flavour because of the production of lactic acid and certain organic acids. They are able to alter the texture of the products, affect the taste and also hydrolyze proteins. Furthermore, starters may help in preservation of final products by the production of bacteriocins in addition to lactic acid (Batt, 2014).

2.2.4. Genus *Lactobacillus*

The genus *Lactobacillus* belongs to the large group of lactic acid bacteria occurring in shapes of rods, cocci or coccobacilli having a DNA base composition of less than 50 mol% of G+C content. The optimal growth temperature is between 30 and 40°C, although some species are able to grow at temperatures higher than 55°C and lower than 2°C. Lactobacilli are acid-tolerant (pH range for growth is between 3 and 8), but minimum pH for growth varies with species and with the acid present in the environment (Pot et al., 2014; Wareing and Fernandes, 2010; Pot and Tsakalidou, 2009).

Based on the fermentation of end products, *Lactobacillus* species are divided into three groups: obligate homofermenters (*L. helveticus, L. acidophilus, L. delbrueckii*), facultative heterofermenters (*L. plantarum* and *L. casei*) and obligate heterofermenters (*L. brevis, L. reuteri, L. fermentum* or *L. kefir*) (Liptáková et al., 2017; Hassan and Frank, 2001). While the fermentative conversion of sugars present in the raw materials into lactic acid is their main function, production of anti-microbial peptides, exopolysaccharides and a variety of other metabolites are other important properties (de Vries et al., 2006).

Lactobacilli may be found in a diverse environments, including nutrient-rich dairy products, microbial-heavy host habitats, as well as natural ecological niches. In humans, lactobacilli are found throughout the gastrointestinal tract, from the oral cavity to faecal material. In the oral cavity, lactobacilli are present in saliva and in dental plaque. A lot of strains are usually isolated from various other sources such as vaginal microbiota or breast milk (Barrangou et al., 2012).

Due to the long history of usage in food fermentations and in the food industry as well as lack of pathogenicity, lactobacilli are “Generally Recognized As Safe” (GRAS) microorganisms (Åvall-Jääskeläinen and Palva, 2005). Lactobacilli are also associated with food production because of the preservative action due to acidification, and/or the modification of the organoleptic characteristics of foods, such as flavour and texture. Various strains of lactobacilli are used as starter cultures for fermentation of dairy products, meat (sausages), fish,

vegetables (olives, sauerkraut), etc. Some lactobacilli isolated from the gastrointestinal tract (GIT) have also been associated with health benefits, which have given rise to their designation as probiotics (Giraffa et al., 2010).

2.2.5. *Lactobacillus plantarum*

L. plantarum is a mesophilic bacterium with the growth occurring at 10-15°C but not at 45°C (Corsetti and Gobbetti, 2003). Cells of *L. plantarum* are straight rods with rounded ends (0.9 ± 1.2 x 3.0 ± 8.0 mm), occurring singly, in pairs or in short chains. *L. plantarum* belongs to the facultative heterofermentative group of lactobacilli, indicating that sugars may be fermented via the EMP pathway or the pentose-phosfate pathway. In media requires vitamins and minerals such as calcium, pantothenate and niacin for growth (Hammes and Vogel, 1995). Some strains are showing atypical characteristics for the genus *Lactobacillus*, such as pseudocatalase activity and nitrate reduction. The G+C content of the DNA is 44 ±46 mol% (Corsetti and Gobbetti, 2003).

Many *Lactobacillus* species are highly specialized and are only found in a limited number of niches (*Lactobacillus delbrueckii* or *Lactobacillus rhamnosus*) (Siezen and van Hylckama Vlieg, 2011). *L. plantarum* has the coding capacity for the uptake and utilization of many different sugars, uptake of peptides and formation of most amino acids. A large number of surface-anchored proteins suggests its potential to associate with many different surfaces and potential substrates for growth. Taken all together, this reflects the potential of *L. plantarum* to grow in a large range of environmental niches (Kleerebezem et al., 2003). *L. plantarum* is highly versatile and found in different dairy products, vegetables, meat, silage, wine, gastrointestinal, vaginal and urogenital tract (Seddik et al., 2017; Siezen and van Hylckama Vlieg, 2011). It is also naturally and frequently present in human breast milk (Collado et al. 2009; Martín et al., 2007). Therefore, *L. plantarum* has several applications in the food industry and has been used as a starter culture in various food fermentation processes contributing to the organoleptic properties. It is one of the most frequent species related with cheese production and plays an important role in ripening (Todorov and Franco, 2010). Furthermore, *L. plantarum* is often the dominating *Lactobacillus spp.* in traditional lactic acid fermented foods based on plant material such as sauerkraut, green olives and cucumbers. The fact that *L. plantarum* frequently predominates in spontaneously lactic acid fermented foods (pH < 4) and is able to survive the passage through the acid conditions of the human stomach, naturally points to its high resistance to acid conditions (Molin, 2008).

Besides producing organic acids, *L. plantarum* is able to compete with microorganisms populating the same food ecosystem by excreting various antimicrobial compounds such as hydrogen peroxide, diacetyl and antimicrobial peptides as bacteriocins (Arena et al., 2016; Corsetti and Gobetti, 2003). Several studies showed that *L. plantarum* exerts inhibitory activity against pathogenic and food spoilage microorganisms such as *Escherichia coli* (Davoodabadi et al., 2015; Tambekar and Bhutada, 2009; Todorov and Dicks, 2005), *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Todorov and Dicks, 2005), *Helicobacter pylori* (Sunanliganon et al., 2012), *Clostridium difficile, Clostridium perfringens* (Schoster et al., 2013), *Listeria monocytogenes, Bacillus cereus, Bacillus subtilis* (Elegado et al., 2004) *Enterococcus faecalis* (Todorov and Dicks, 2005; Elegado et al., 2004), *Salmonella* Typhimurium (Potočnjak et al., 2017), *Klebsiella pneumoniae* (Tambekar and Bhutada, 2009), *Shigella flexneri* (Davoodabadi et al., 2015; Tambekar and Bhutada, 2009), *Yersinia enterocolitica* (Davoodabadi et al., 2015). Because of the antagonistic feature, some *L. plantarum* strains are used in food preservation with the extension of shelf life and reducing or even replacing chemical additives, or they can be used as supporting therapeutic agents in the treatment of infections caused by susceptible microorganisms (Dinev et al., 2017; Arena et al., 2016).

A variety of *L. plantarum* strains are also applied as probiotics. Among the lactic acid bacteria, *L. plantarum* attracts many researchers because of its wide range of applications in the medical field with antioxidant, anticancer, anti-inflammatory, antiproliferative, antiobesity and antidiabetic properties (Arasu et al., 2015). Certain studies have shown that among the other effects, consumption of *L. plantarum* reduced carriage of faecal *Enterobacteriaceae* (Kingamkono et al., 1999), kidney stones (Sasikumar et al., 2014) and symptoms of irritable bowel syndrome (IBS), such as pain and flatulence (Niedzielin et al., 2001; Nobaek et al., 2000). *L. plantarum* also showed cholesterol-lowering effects on animal models (Guan et al., 2017; Li et al., 2014; Nguyen et al., 2007), as well as in patients (Naruszewicz et al., 2002; Bukowska, 1998) leading to decreased certain risk factors for coronary artery disease. Furthermore, intake of *L. plantarum* may have a preventive effect on gastrointestinal symptoms during antibiotic treatment (Lönnermark et al., 2010). However, it has been observed that there is a significant strain-specific variability in the functional probiotic properties of *L. plantarum* (Strahinic et al., 2007).

2.3. GROWTH AND REPRODUCTION OF BACTERIA

Growth in the microbial world usually refers to an increase in the numbers of individuals; that represents an increase in the population size (Pommerville, 2013). Bacteria are generally reproducing by the process of binary fission, resulting into two daughter cells of equal sizes. A cell growth is characterized by increasing in size, during which time the amount of each new cell component is doubled and the genome is replicated (Posten and Cooney, 1993).

Several factors related to the environmental conditions influence the growth of microorganisms. These factors may be divided into intrinsic and extrinsic elements. The most important intrinsic factors represent pH, redox potential, water activity and the presence of antimicrobial substances. These factors may influence the capacity by stimulating or retarding the growth of microorganisms. Extrinsic factors such as temperature, atmosphere and relative humidity represent other critical factors affecting the growth and reproduction (Wareing and Fernandes, 2010).

The increase in numbers of bacterial mass may be measured as a function of time to obtain a growth curve. The classical growth curve consists of four phases: the lag phase, the logarithmic phase, the stationary phase and the decline phase. The first part of the growth curve represents an adaptation of bacteria to the new environmental and nutritional conditions. After this preparation phase, cell numbers begin to increase and the population enters an active, exponential stage of growth called the logarithmic (log) phase. In this phase, cells undergo binary fission and the generation time is dependent on the species and environmental conditions presented. After some time, as a result of metabolic activity and growth of microorganism, available nutrients are limited and waste products are accumulating. This state leads to a decline in the growth rate. The growth finishes as the cells enter the stationary phase of growth, when the viable cells equal the number of nonviable or dead cells. Finally, if the nutrients in external environment remain limited or the quantities become exceedingly low, cells start to lose their viability or the ability to form colonies and the culture enters a decline (death) phase (Pommerville, 2013; Cooper, 2004).

2.3.1. Predictive microbiology

Predictive food microbiology is an emerging multidisciplinary area of food microbiology including disciplines such as mathematics, microbiology, engineering and chemistry in order to develop and apply mathematical models predicting the responses of microorganisms to specified environmental variables (McDonald and Sun, 1999). It has a wide range of practical applications in improving microbial food safety and quality of final products

and is the basis for the development of quantitative microbial risk assessment (Pérez-Rodríguez, 2014; Fakruddin et al., 2011).

A predictive food microbiology model is a mathematical expression describing the growth, survival, inactivation, or metabolic activities of foodborne microorganism (Buchanan and Whiting, 1997). Within that broad definition, several different classification schemes are possible to group the models. The most often used classification is the distinction between primary, secondary and tertiary models (Devlieghere et al., 2009).

2.3.1.1. Baranyi's model

The growth model of Baranyi and Roberts (1994) is one of the most used primary models describing changes in the population of bacteria in dependence to time when placed into a single environment (Buchanan and Whiting, 1997). The model of Baranyi and Roberts is widely used because it is applicable under dynamic environmental conditions, it has a good fitting capacity and majority of the model parameters are biologically interpretable (Van Impe et al., 2005). The explicit form of the model is described with following equation:

$$y(t) = y_0 + \mu_{max}t + \frac{1}{\mu_{max}}\ln(e^{-v\cdot t} + e^{-h_0} - e^{-v\cdot t-h_0}) - \frac{1}{m}\ln\left(1 + \frac{e^{m\mu_{max}t} + \frac{1}{\mu_{max}}\ln(e^{-v\cdot t} + e^{-h_0} - e^{-v\cdot t-h_0} - 1}{e^{m(y_{max}-y_0)}}\right)$$

where $y(t) = \ln(x(t))$ with $x(t)$ the cell concentration (CFU mL^{-1}), $y_0 = \ln(x_0)$, $y_{max} = \ln(x_{max})$, x_0 - initial and x_{max} the asymptotic cell concentration, μ_{max} is the maximum specific growth rate (h^{-1}), m is a curvature parameter to characterize the transition from the exponential phase, v is a curvature parameter to characterize the transition to the exponential phase, h_0 is a dimensionless parameter quantifying the initial physiological state of the cells (the lag time λ (h) can be calculated as $\frac{h_0}{\mu_{max}}$). It is suggested that $v = \mu_{max}$, $m = 1$, so the final model has four parameters: y_0, y_{max}, h_0 and μ_{max} (Grijspeerdt and Vanrolleghem, 1999).

2.3.1.2. Secondary models

Secondary models describe the effect of environmental factors on certain kinetic parameters, particularly the growth rate and lag time duration. The most important group of models within predictive microbiology are square root models and cardinal-type models (Pérez-Rodríguez, 2014).

Square root models were initially proposed by Ratkowsky et al. (1982), who observed a linear relationship between the square root of the maximum growth rate and temperature (at suboptimal conditions for growth):

$$\sqrt{\mu_{\max}} = b \cdot (T - T_{\min})$$

where $T_{\min}$ is the notional minimum temperature below which maximum growth rate is equal 0 (it ranges between 2°C and 3°C below the observed minimum temperature) (Pérez-Rodríguez and Valero, 2013). Later, this model was extended to cover the whole temperature growth range (Ratkowsky et al., 1983):

$$\sqrt{\mu_{\max}} = b \cdot (T - T_{\min})\{1 - \exp[c(T - T_{\max})]\}$$

The first cardinal model was developed by Rosso et al. (1995), called the cardinal temperature and pH model (CTPM). It is describing the effect of temperature and pH on the growth rate of microorganism, based on the cardinal values: the optimal, the minimal, and the maximal temperature and pH at which growth is possible according to the equations:

$$\mu_{max} = \frac{\mu_{opt}(T - T_{\max})(T - T_{\min})^2}{(T_{\text{opt}} - T_{\min})\left[(T_{\text{opt}} - T_{\min})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max})(T_{\text{opt}} + T_{\min} - 2T)\right]}$$

$$\mu_{max} = \frac{\mu_{opt}(\text{pH} - \text{pH}_{\min})(\text{pH} - \text{pH}_{\max})}{(\text{pH} - \text{pH}_{\min})(\text{pH} - \text{pH}_{\max}) - (\text{pH} - \text{pH}_{\text{opt}})^2}$$

3. EXPERIMENTAL PART

3.1. MICROORGANISMS

The potentially probiotic *Lactobacillus plantarum* was isolated from breast milk and identification was provided by the Food Research Institute in Bratislava, Slovakia (Liptáková et al., 2016). The isolate of *L. plantarum* was maintained in de Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) at 6 ± 0.5°C.

Fresco DVS 1010 culture (consists of *Lactococcus lactis* spp. *lactis*, *Lactococcus lactis* spp. *cremoris*, *Streptococcus salivarius* spp. *thermophilus*) is a commercial culture from Christian and Hansen (Hørsholm, Denmark) kindly provided by Rajo a.s. (Bratislava, Slovakia) and was kept in a freezer.

3.2. CHEMICALS AND SOLUTIONS

- Saline solution

ISO 6887-1:1999 recommends saline solution as a diluent for the preparation of initial suspension for microbiological samples. Serial ten-fold dilutions were prepared in a solution of 0.85% NaCl (w/v) and 0.1% (w/v) of peptone (Biolife, Milan, Italy). After adjusting pH (7.0 ± 0.2), 9 ml of diluent was poured into each test-tube. The sterilization was conducted by autoclaving for 20 minutes at 121°C under the pressure of 120 kPa. Unused sterile saline solution was stored at 5 ± 0.5°C until further use.

- MRS broth

MRS broth (Biokar Diagnostics, Beauvais, France) was used for storage and cultivation of *Lactobacillus plantarum*. The pH was adjusted to 6.4 ± 0.2 and 9 ml of MRS broth was poured into test-tubes and sterilized for 20 minutes at 121°C under pressure of 120 kPa. After sterilization and cooling, MRS broth was stored at 5 ± 0.5°C.

- MRS agar

MRS agar (Merck, Darmstadt, Germany) was used for determination of the numbers of *Lactobacillus plantarum* in lactose-free milk. After adjusting the pH to 5.7 ± 0.2, the media was sterilized by autoclaving for 20 minutes at 121°C (120 kPa). The unused media was stored in a fridge at 5 ± 0.5°C.

- Vegitone MRS agar

Vegitone MRS agar (Sigma-Aldrich Chemie GmbH, Switzerland) was used as a selective media for enumeration of *Lactobacillus plantarum* in prepared soya substrates. After addition of 0.1 ml of Tween 80 (Biolife, Milan, Italy), the pH was adjusted to 5.5 ± 0.2 followed

with sterilization in autoclave for 20 minutes at 121°C (120 kPa). The media that was not used immediatelly was kept in a fridge (5 ± 0.5°C) until further use.

- M17 broth

For preparation of 24 h culture of Fresco DVS 1010, M17 broth (Biokar Diagnostics, Beauvais, France) was used. The pH was adjusted to 7.1 ± 0.1. The media was sterilized for 20 minutes in autoclave (121°C, 120 kPa). After sterilization and cooling, it was stored at 5 ± 0.5°C.

- M17 agar

M17 agar (Biokar Diagnostics, Beauvais, France) was used for the enumeration of microorganisms of mixed Fresco DVS 1010 culture. After pH adjustment (7.1 ± 0.1), the medium was sterilized at 121°C and 120 kPa for 20 minutes in autoclave. The unused media was kept at 5 ± 0.5°C.

- Solutions for pH adjusting
  - 1 mol/L or 5 mol/L hydrochloric acid (HCl)
  - 1 mol/L or 5 mol/L sodium hydroxide (NaOH)

3.3. FERMENTATION SUBSTRATES

- Soya flour

Soya substrates were prepared from soya flour obtained from mill house (Mlyn Zrno, Šišov, Slovakia).

- Flavouring ingredients
  - Sucrose (Sigma Aldrich, Steinheim, Germany)
  - Chocolate flavour (Rajo a.s., Bratislava, Slovakia): 26.5% chocolate powder (32% cocoa powder, 68% sucrose), 24% sucrose, 10% glucose-fructose syrup, modified starch, xanthan gum, vanillin and water
  - Caramel flavour (Rajo a.s., Bratislava, Slovakia): 35% caramel syrup (invert sugar syrup, whole milk powder, sugar, water, butter), 10% glucose-fructose syrup, 28.5% sucrose, modified starch (maize), burned sugar and water

- Milk and lactose-free milk

Ultra-high-temperature processed (UHT) cow's milk and lactose-free cow's milk with 1.5% fat content were obtained from Rajo a.s., Bratislava, Slovakia.

- Distilled water

3.4. LABORATORY EQUIPMENT

- Autoclave Timo 88944, PBI International, Milan, Italy
- Automatic pipettes 10 to 1000 ml, Eppendorf, Hamburg, Germany
- Centrifuge EBA 20 HettichLab, Tuttlingen, Germany
- Digital scale Kern 572, Balingen, Germany
- pH meter inoLab pH 720, WTW Weilheim, Germany
- pH meter with injection electrode Portamess Knick, Berlin, Germany
- Thermostats
- Vortex Reax top, Schwabach, Germany
- Bunsen burner
- Glassware

3.5. PROCEDURE

3.5.1. Preparation of the starter culture

The isolate *L. plantarum* was first sub-cultured three times for 24 h at 37 ± 0.5°C (5% CO_2) in de Man, Rogosa and Sharpe (MRS) broth from the frozen stock containing MRS broth and 25% glycerol before using it as an inoculum (stored at -30°C). The starter culture was obtained by overnight incubation at 37 ± 0.5°C (5% CO_2) in MRS broth.

Fresco DVS 1010 culture was kept in a deep-freezer (-20°C). 1 cm^3 of culture was suspended into 100 ml of M17 broth and cultivated aerobically overnight at 30 ± 0.5°C.

Pure 24 h cultures of studied lactic acid bacteria were centrifuged at 6000 rpm for 5 minutes. The cultures were washed in 10 mL of sterile distilled water and again centrifuged under the same conditions. After centrifugation, supernatant was decanted and pellets were resuspended in distilled water to its original volume (Angelov et al., 2006).

3.5.2. Soya substrates: preparation, fermentation and storage

Soya substrates were prepared from soya flour, sucrose (2%) and water (milk) or lactose-free milk (fat content 1.5%). The consistency of the substrates was adjusted to the consistency of a yogurt, making it suitable for eating with a spoon. In milk-based substrate, the content of soya flour was 18% (w/v) while 80% was milk. To maintain the same consistency, the content of flour in water-based and lactose-free milk-based substrate was 25% (w/v), while 73% was water or lactose-free milk.

Flavoured soya substrates were prepared by substitution of sucrose with chocolate or caramel flavour, added right after the sterilization. The ratio of soya substrate and flavouring

compounds was 80% : 20%. In case of milk substrates, the content of soya flour was 18% (w/v), in lactose-free milk substrates 20% (w/v) and in those prepared of water, 25% (w/v), in order to maintain the same consistency. Totally, nine different kinds of soya substrates were prepared.

After sterile weighing the components into the beakers, soya substrates were boiled at 100°C for 20 minutes while at the same time were stirred. Afterwards, they were sterilized in the autoclave for 20 minutes at 121°C under the pressure of 120 kPa. In case of lactose-free milk substrates sterilization was conducted for 30 minutes at 100°C. Subsequently, they were cooled down and 20% of flavour (chocolate or caramel) was added.

The fermentation process started by inoculating 5% of starter mixed Fresco DVS 1010 culture in order to achieve approximately 10^5 to 10^6 CFU mL^{-1}. After the addition of starter culture, static anaerobic fermentation was carried out for 8 hours at 37 ± 0.5°C (5% CO_2). Every 2 hours, 1 mL of samples were taken for analyses of the counts. Potentially probiotic isolate *L. plantarum* was inoculated to the substrates after the fermentation process was completed in densities of approximately 10^8 CFU mL^{-1}. Subsequently, final products were stored at 6 ± 0.5°C for 21 days. Each day 1 mL of the sample was taken for analysis of counts of studied lactic acid bacteria. The decrease of pH was monitored every second day. All experiments were carried out in duplicate while the sample for measuring pH was taken from only one parallel beaker.

3.5.3. *L. plantarum* in lactose free milk: substrate inoculation and cultivation conditions

The standard 24 h suspension of *Lactobacillus plantarum* in MRS broth was inoculated to the ultra-high-temperature processed (UHT) lactose-free milk to achieve concentration of approximately 10^3 CFU mL^{-1}. Two parallel static cultivations of lactose-free milk samples were carried out at temperatures from 8 to 40 ± 0.5°C under aerobic conditions. In an appropriate time intervals, depending on the incubation temperature, 1 mL of sample was taken for analysis of the counts of *L. plantarum*. At lower temperatures time intervals were longer (for example at temperature of 8°C samples were taken once a day), while at higher temperatures they were much shorter (at 37°C samples were taken every 2-3 hours).

3.6. METHODS

3.6.1. Bacteriological analyses

During the fermentation process and cold storage, the growth of lactic acid bacteria was determined in accordance to the standard STN ISO 15124. Total viable counts of *Lactobacillus plantarum* in soya substrates were determined after appropriate dilution and cultivation on Vegitone MRS agar plates under anaerobic conditions at 37 ± 0.5°C (5% CO_2) for 48 hours, while presumptive numbers of *L. plantarum* in lactose-free milk were estimated using MRS

agar under the same conditions. Counts of Fresco DVS 1010 culture were determined after cultivation on M17 agar plates under aerobic conditions 30 ± 0.5°C for 24 hours.

3.6.2. Enumeration of microorganisms

The total numbers of microorganisms in a sample were calculated using following mathematical equation:

$$N = \frac{\sum C}{V \cdot (n_1 + 0.1n_2) \cdot d}$$

where N is the number of microorganisms (CFU mL^{-1}), $\sum C$ is the sum of the colonies counted on the Petri dishes, n_1 is the number of dishes from the first dilution, n_2 the number of dishes from the second dilution used for the calculation, d is the dilution factor and V is the volume of inoculum inoculated on Petri dish.

3.6.3. Evaluation of growth parameters

Growth curves of *Lactobacillus plantarum* for each temperature in inoculated UHT lactose-free milk and growth curves of studied lactic acid bacteria in soya substrates were built separately by fitting data to the Baranyi model (Baranyi and Roberts, 1994) using DMFit. Growth and metabolic parameters were calculated from each growth curve, from two parallel experiments. Growth rate as a function of suboptimal growth temperature in lactose-free milk has been described by Ratkowsky square root model (Ratkowsky et al., 1983), while cardinal temperatures were obtained using cardinal temperature model with inflection (CTMI) (Rosso et al., 1995).

3.6.4. Statistical analysis

Obtained growth parameters of studied lactic acid bacteria were analyzed using Microsoft Excel 2013 (Microsoft, Redmond, USA). Data were treated by Student t-test with a least significant difference of 95%.

4. RESULTS AND DISCUSSION

4.1. GROWTH OF *Lactobacillus plantarum* IN LACTOSE-FREE MILK

Growth trials with *Lactobacillus plantarum* in UHT lactose-free milk were performed at 8, 12, 15, 18, 21, 25, 30, 34, 37, 40 ± 0.5°C. A temperature range was selected in order to detect the entire growth ability of the microorganism.

During the growth and multiplication of *L. plantarum*, the decrease of pH from initial values about 0.17-1.16 units in dependence on the cultivation temperature was observed. Observed and calculated parameters of pH are, for better interpretation, shown in Table 1.

Table 1. The effect of temperature on pH values in lactose-free milk

Temperature (°C)	**pH_0**	**pH_{end}**	**k_{pH} (h^{-1})**
8	6.51	6.61	0.0002
12	6.45	6.28	-0.0005
15	6.54	6.59	0.0002
18	6.55	6.14	-0.004
21	6.54	6.36	-0.0025
25	6.49	5.97	-0.0055
30	6.44	6.14	-0.0081
34	6.44	5.97	-0.0113
37	6.51	5.99	-0.1535
40	6.49	5.33	-0.0247

(pH_0- initial pH value, pH_{end}- final pH value, k_{pH}- rate constant for the decrease of pH)

In a study of Matejčeková et al. (2016a), in case of UHT milk (3.5% fat) during the growth and multiplication of *L. plantarum* no significant changes of pH values (0.00-0.24 units) were reported. This can be explained by the low ability of *L. plantarum* to utilize lactose and convert pyruvate to lactate in a rate to match the glycolysis (Jyoti et al., 2004). However, in lactose-free milk, disaccharide lactose is broken down during processing into two simple sugars, galactose and glucose. This is the reason why metabolic activity of *L. plantarum* is higher in lactose-free milk and, thus, pH decreased for more units. The fastest decrease of pH

was reported at 37°C (-0.1535 h^{-1}). In their study, Smetanková et al. (2012) reported pH values below 4.3 during growth of *L. plantarum* in MRS broth at temperatures of 30, 37, 45°C with a faster decrease of pH under aerobic than under anaerobic conditions in most cases. Salmerón et al. (2014) evaluated the growth and metabolism of *L. plantarum* in cereal beverages (oat, barley and malt substrates), where pH values after 10 h of fermentation were below 3.7.

In a model environment isolate *L. plantarum* showed good growth with the growth rates ranging between 0.017 to 0.168 log CFU mL^{-1} h^{-1} (Tab. 2). Growth curves at all temperatures are shown in Figure 1 and calculated growth parameters are for better interpretation summarized in Table 2.

Table 2. Growth characteristics of *L. plantarum* with respect to incubation temperature

Temperature (°C)	**Gr (log CFU mL^{-1} h^{-1})**	**N_0 (log CFU mL^{-1})**	**N_{max} (log CFU mL^{-1})**	**λ (h)**	**t_d (h)**
8	-0.0015	4.10	3.94	-	-
12	0.0174	3.24	7.56	78.0	17.3
15	0.0295	4.03	7.65	17.6	10.2
18	0.0517	3.28	7.75	13.5	5.8
21	0.0752	2.66	7.94	-	4.0
25	0.1130	3.03	7.81	2.0	2.7
30	0.1675	3.17	7.64	1.5	1.8
34	0.1684	3.17	7.67	-	1.8
37	0.1601	3.36	7.42	-	1.9
40	0.0804	3.22	7.55	-	3.7

(Gr- growth rate, λ- lag phase duration, t_d-time to double, N_0- initial numbers of *L. plantarum*, N_{max}- numbers of *L. plantarum* in stationary phase)

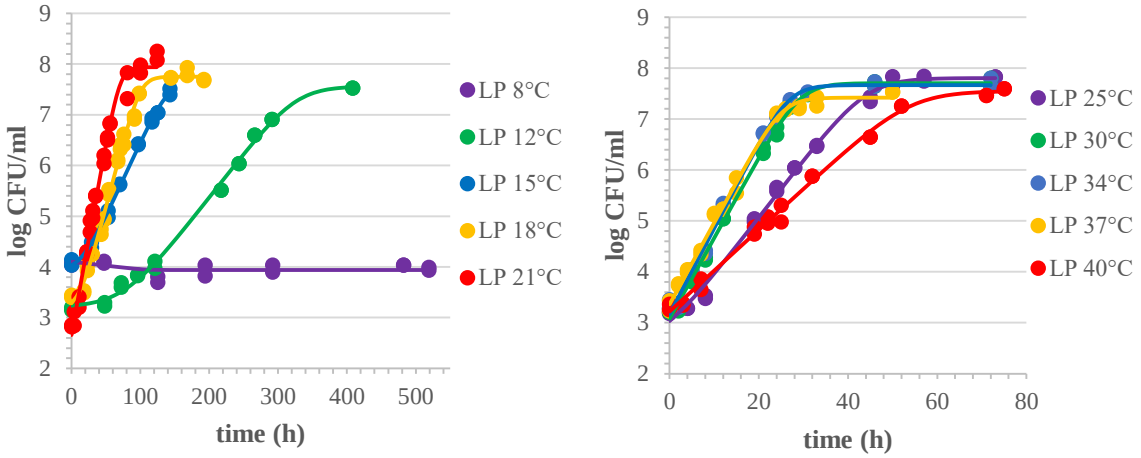

Figure 1. Growth dynamics of *Lactobacillus plantarum* in lactose-free milk in depedence to temperature

In the stationary phase, *L. plantarum* reached counts in average 10^7 CFU mL^{-1} from the initial 10^3 CFU mL^{-1} at all studied temperatures (except for marginal 8°C). At the lowest temperature (8°C) growth of *L. plantarum* was not observed. At these conditions, the isolate was not able to adapt to the environmental conditions even after 21 days of incubation period. On the contrary, a slight decrease in cell numbers occured in case of *L. rhamnosus* GG (Gr = 0.030 log CFU mL^{-1} h^{-1}) at same temperature in UHT milk that reached stationary phase after 9 days of incubation (Valík et al., 2008). Increasing of the incubation temperature by 4°C (12°C) resulted in the growth of studied isolate. The growth was still slow, represented by time to double of 17.3 h and the stationary phase was reached on the 14th day of incubation. Aerobic cultivation of *L. plantarum* at 15°C decreased lag phase duration 4.4 times in comparison with that at 12°C, while the growth rate was 69% faster. At 15°C, growth rate of *L. plantarum* in lactose-free milk was shown to be the same as the growth rate of *L. rhamnosus* VT1 in milk (Liptáková et al., 2007). At 18°C, *L. plantarum* reached stationary phase after 6 days of incubation, while the maximal counts in stationary phase reached were 7.8 log CFU mL^{-1}. In a study of Matejčeková et al. (2016a) the same results of *L. plantarum* in milk were obtained. Further increase of incubation temperature led to the increase of the growth rate while the lag phase duration and time to double was shortened, as well as the time to reach the stationary phase. At 37°C, the shortest time necessary to reach the stationary phase was achieved (33 h). Maximal growth rates were observed at temperatures of 30 and 34°C, while at 37°C the growth rate was lower only 5%. The growth rate of *L. plantarum* at 37°C (Gr = 0.1601 log CFU mL^{-1}) was characterized as two times slower than the growth rate of *L. acidophilus* in UHT milk at the same temperature (Gr = 0.335 log CFU mL^{-1}) (Medveďová et al., 2016). The fastest growth

was also calculated in case of *L. paracasei* subsp. *paracasei* (Gr = 0.201 log CFU mL^{-1}) in milk (Pelikánová et al., 2011). Optimal temperature calculated for studied isolate in UHT milk is 34.7°C (Matejčeková et al., 2016a). In the last selected temperature (40°C) the growth rate decreased by 50% in comparison to 37°C and *L. plantarum* reached stationary phase after 2.5 days of incubation. On the contrary, *L. rhamnosus* GG demonstrated about 91% higher growth rate in milk at 41°C (G_r = 0.859 log CFU mL^{-1}) (Valík et al., 2008).

At suboptimal course, the temperature influence on the growth rate is characterized by Ratkowsky square root model (Fig. 2) that linearizes the dependence of the growth rate on the incubation temperature:

$$\sqrt{Gr} = 0.014.\ T + 0.0719\ R^2 = 0.9736$$

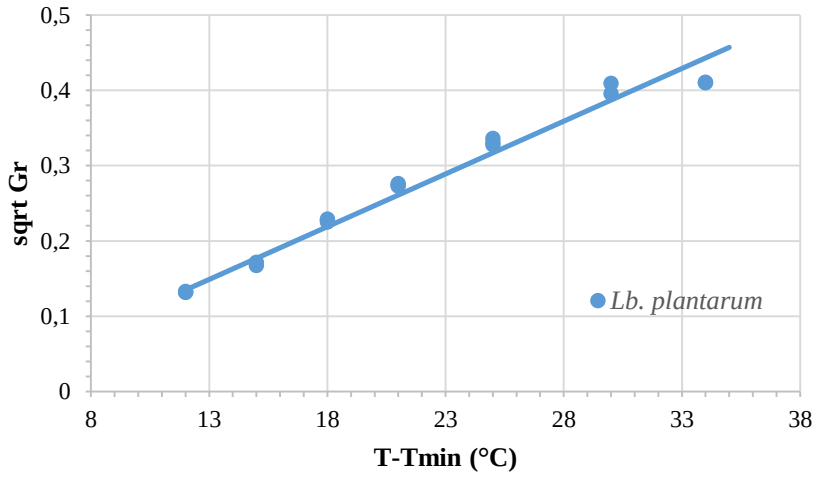

Figure 2. Ratkowsky model as applied to the growth rate (Gr) of *L. plantarum*

Finally, by using the CTMI model, the optimal temperature (T_{opt}) for *L. plantarum* growth in UHT lactose-free milk of 33.7°C was calculated. Under the optimal temperature conditions, the maximal growth rate (Gr_{opt}) would be 0.167 log CFU mL^{-1} h^{-1}. Moreover, other cardinal temperatures were estimated: theoretical minimal temperature (T_{min}) below which no growth occurs is 7.8°C and the maximal temperature (T_{max}) that allows *L. plantarum* to grow is 41°C.

4.2. FERMENTATION OF SOYA SUBSTRATES

The success of new probiotic formulations does not rely only on the ability to provide sufficient amount of probiotic cells reaching the human gastrointestinal tract. A careful strain selection to efficiently control the metabolic end products, the choice and composition of the substrate, final pH, flavour, aroma and texture represent other critical factors in fermentation technologies (De Vuyst, 2000). Consequently, soy products were selected as a substrate for lactic acid fermentation and vehicles for potentially probiotic isolate. However, the food industries always prefer short fermentation periods in order to increase plant output and reduce microbial contamination. A potential solution to this problem is using mixed cultures or co-cultures (Macedo et al., 1999). Thus, based on the above facts and the previous research in this field (Matejčeková et al., 2015; Matejčeková et al., 2016b; Matejčeková et al., 2017), mixed Fresco DVS 1010 culture was used for fermentation of prepared substrates and human derived potentially probiotic isolate *L. plantarum* was tested for the ability to survive throughout the specified shelf life of final products. Short fermentation time (8 h) was preferable in order to minimize the risk of contamination.

4.2.1. Growth of Fresco DVS 1010 culture and survival of *Lactobacillus plantarum* in flavourless soya substrates

The results of cell growth of cocci from Fresco DVS 1010 culture and *Lactobacillus plantarum* survival in soya substrates based on milk, water and lactose-free milk, respectively, are shown in Figures 3, 4, and 5. In milk-based substrate, mixed Fresco DVS 1010 culture entered immediately the exponential phase of growth with rate 0.553 log CFU mL^{-1} h^{-1}. Viability of counts was maintained throughout the cold storage for 21 days at 6 ± 0.5°C. During the fermentation process, the application of 5% (v/v) starter culture resulted in reaching the pH level of 5 with the rate -0.293 h^{-1} that during cold storage decreased for another 0.3 units to the final value of 4.7. Initial counts of *L. plantarum* decreased about 1 log order at 6 ± 0.5°C during storage period of 21 days (rate of decrease -0.0009 log CFU mL^{-1} h^{-1}) to final counts $N_{end} = 6.7 \times 10^8$ CFU mL^{-1}.

In case of water based soya substrate, Fresco DVS 1010 culture showed similar growth rate (0.533 log CFU mL^{-1} h^{-1}), but during the storage period, started to decrease after 448 hours at a rate of -0.0042 log CFU mL^{-1} h^{-1}. After 8 hours of fermentation, measured pH showed a decrease of 1.09 units with a rate of -0.276 h^{-1} and at the end of storage time, pH of 4.61 was measured. The concentration of added *L. plantarum* was maintained in numbers added after the

fermentation process ($N_0 = 1.8 \times 10^9$ CFU mL^{-1}) during 415 hours. Subsequently, counts dropped about 2 log orders with a rate of -0.0116 log CFU mL^{-1} h^{-1}.

During the fermentation of lactose-free milk soya substrate, the growth rate of Fresco DVS 1010 culture was characterized about 17% and 14% lower than that reached in milk- and water-based, respectively (Gr = 0.459 log CFU mL^{-1} h^{-1}). Throughout the cold storage period (6 ± 0.5°C), initial numbers of the cocci from Fresco DVS 1010 culture decreased about 1 log order. The reduction in pH value was almost the same as in a case of milk-based substrate during the fermentation (a drop of 1.14 units) with a rate of decrease -0.282 h^{-1}. Added initial concentration of *L. plantarum* slightly decreased (-0.0006 log CFU mL^{-1} h^{-1}) to the final concentration $N_{end} = 9.0 \times 10^8$ CFU mL^{-1}.

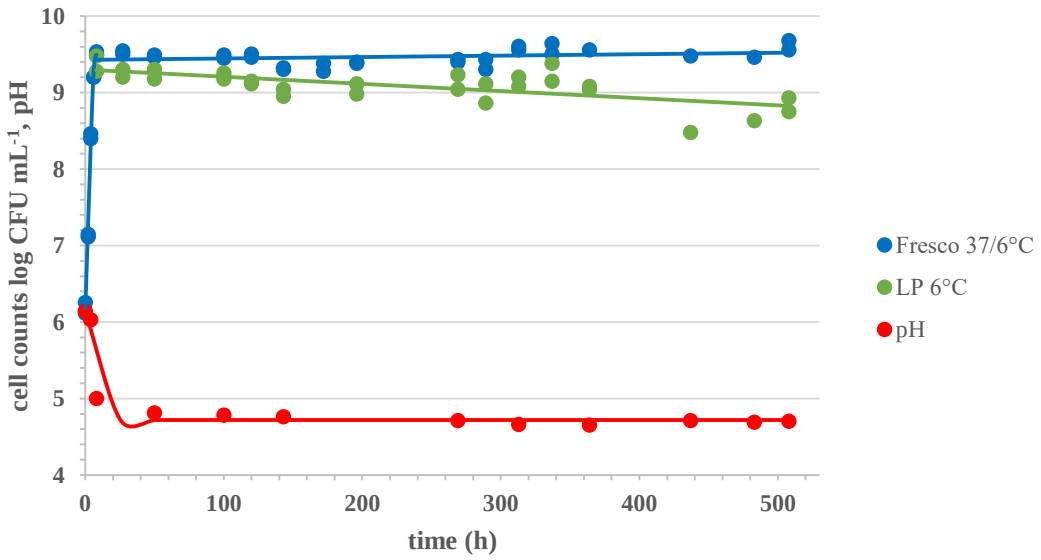

Figure 3. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in milk-based soya substrate during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

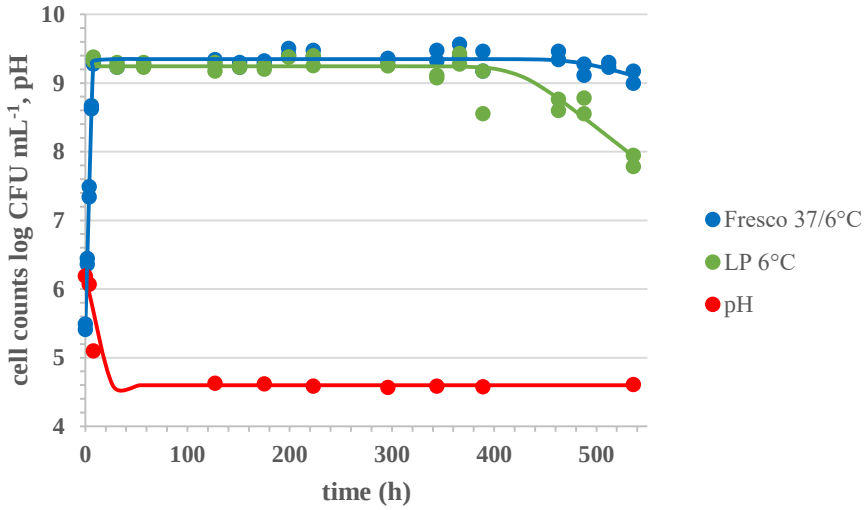

Figure 4. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in water-based soya substrate during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

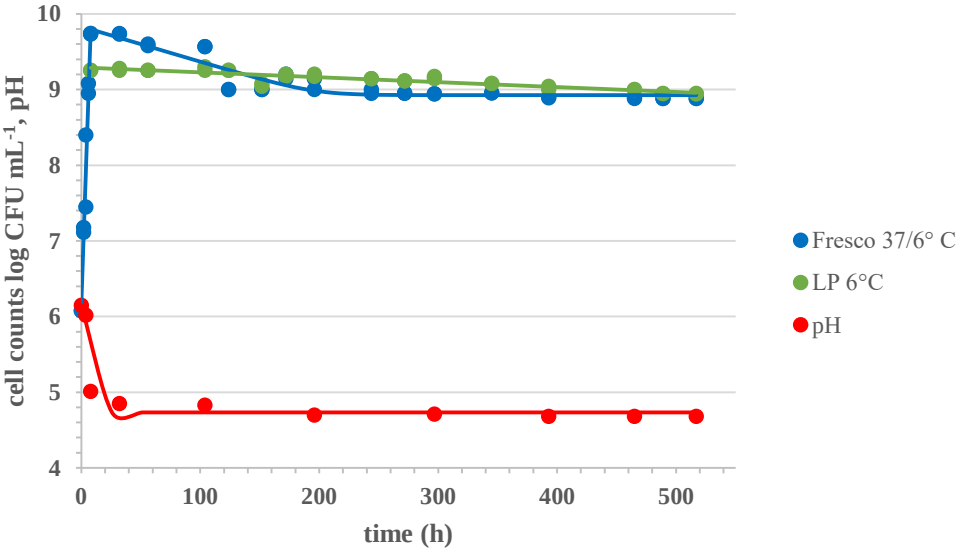

Figure 5. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in lactose-free milk soya substrate during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

4.2.2. Growth of Fresco DVS 1010 culture and *Lactobacillus plantarum* in soya substrates with caramel flavour

The results of cell growth of starter mixed Fresco culture and survival of *L. plantarum* in prepared soya substrates are shown in Figures 6, 7 and 8, where sucrose was replaced by 20% of caramel flavour. Fresco culture in milk-based caramel substrate entered the exponential phase of growth after 39 minutes of lag phase with the rate 0.595 log CFU mL^{-1} h^{-1}. After the fermentation, its viability was preserved during an entire cold storage period of 21 days. The rate of pH drop was set to -0.272 h^{-1} with final pH 4.54 that represents a decrease of about 1.61 units. The initial concentration of *L. plantarum* added right after the fermentation was maintained during almost entire storage period (almost 20 days). Afterwards, numbers of potentially probiotic isolate started to decrease (-0.0242 log CFU mL^{-1} h^{-1}) to the final concentration $N_{end} = 5.0 \times 10^7$ CFU mL^{-1} representing decrease for 1 log order.

In prepared water-based caramel substrate Fresco DVS 1010 culture entered immediately the exponential phase of growth with a rate 0.416 log CFU mL^{-1} h^{-1} that is about 30% lower in comparison to the milk-based. All the monitored microorganisms preserved their viability during 3 weeks of cold storage at 6 ± 0.5 °C with final concentration $N_{end} = 2.5 \times 10^9$ CFU mL^{-1}. The pH decrease during the fermentation process was recorded about 1.01 units from initial value 6.18, which during cold storage decreased furthermore for another 0.48 units to a final value of 4.69.

In case of lactose-free milk caramel soya substrate, Fresco culture showed faster growth rate in comparison to water-based substrate, but still lower than in milk-based substrate (G_r = 0.473 log CFU mL^{-1} h^{-1}). After 106.2 hours of cold storage its concentration started to decrease, that by the end of the storage resulted in the reduction about 1 log order. During the fermentation period, the pH has changed about 1.11 unit (-0.034 h^{-1}), but during storage, a slight decrease was recorded to final value 4.5 (1.65 units). Also a slight decrease in counts of added *L. plantarum* was observed (-0.0008 log CFU mL^{-1} h^{-1}) to the final number $N_{end} = 8.1 \times 10^8$ CFU mL^{-1}.

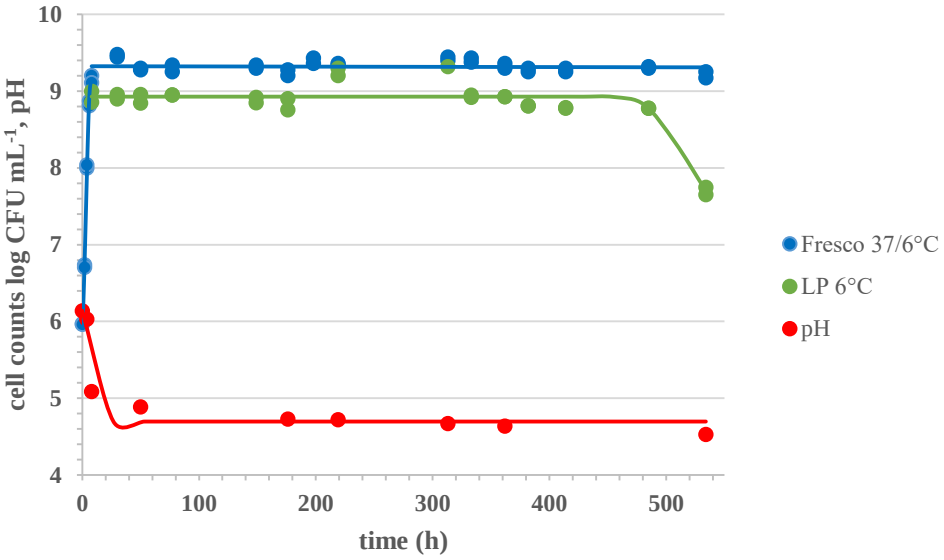

Figure 6. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in milk-based soya substrate with added caramel during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

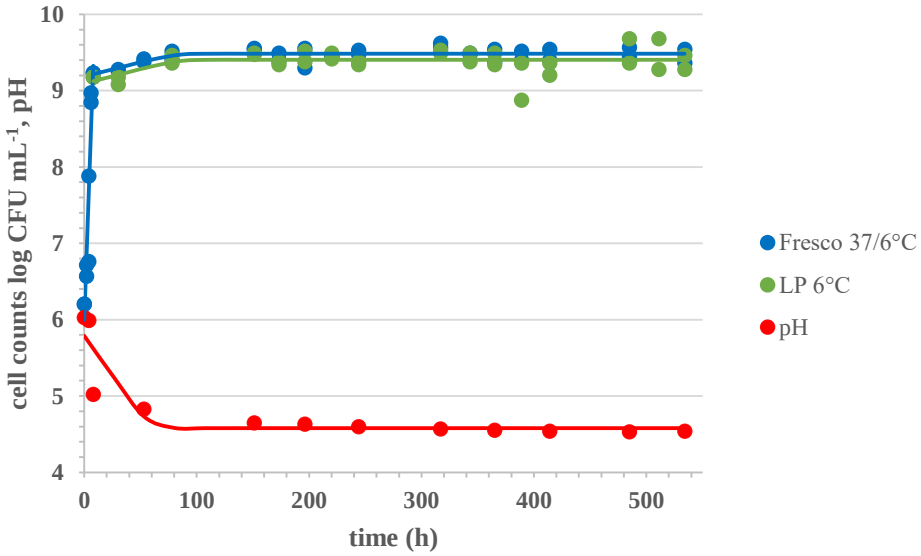

Figure 7. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in water-based soya substrate with added caramel during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

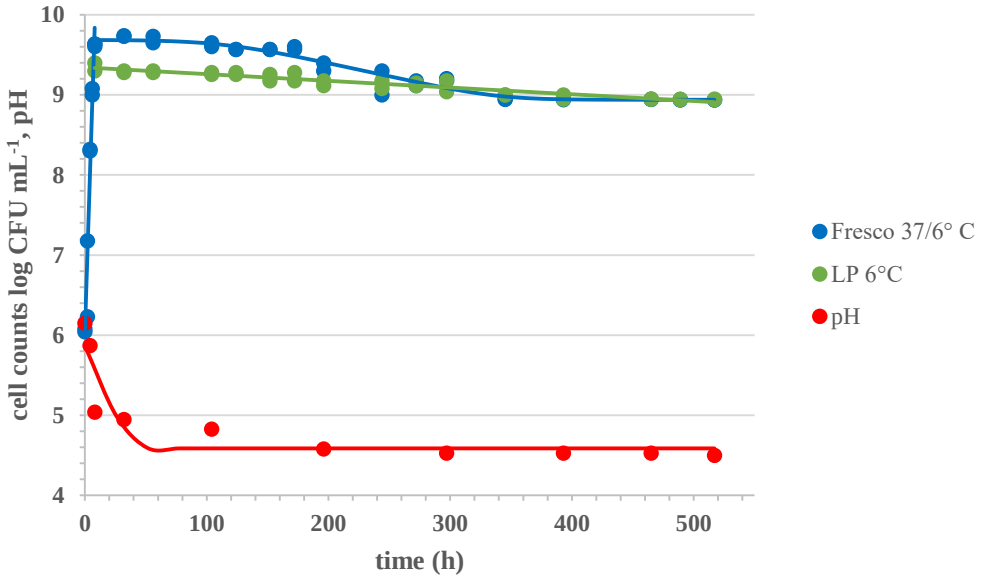

Figure 8. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in lactose-free milk soya substrate with added caramel during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

4.2.3. Growth of Fresco DVS 1010 culture and *Lactobacillus plantarum* in soya substrates with chocolate flavour

Figures 9, 10 and 11 represent growth dynamics of Fresco DVS 1010 culture and viability of *Lactobacillus plantarum* in soya substrates with added chocolate flavour (20%). Fresco DVS 1010 culture in chocolate substrates entered the exponential phase of growth after 52 minutes of lag phase duration in milk-based chocolate substrate, and after 81 minutes, in the case of water-based chocolate product, while in lactose-free milk substrate Fresco entered immediately the exponential phase of growth. The fastest growth rate of Fresco culture was recorded in chocolate substrate based on water (G_r = 0.714 log CFU mL^{-1} h^{-1}), representing an increase of about 27% and 48% in comparison to the growth rate in milk- and lactose-free milk-based substrate, respectively. During the storage period, the viability of Fresco culture was maintained in milk- and water-based substrates, while in lactose-free milk the numbers of the cocci from Fresco culture started to decrease after 199 hours of cold storage and, at the end, were reduced about 1 log order.

During 21 days of cold storage at 6 ± 0.5°C, potentially probiotic isolate *L. plantarum* showed different behaviour depending on the media composition. In milk-based substrate,

added concentration of *L. plantarum* was maintained during almost 297 hours (12 days), afterwards a slight decrease was recorded with a rate -0.0431 log CFU mL^{-1} $h^{-1}$to the final value $N_{end} = 3.6 \times 10^8$ CFU mL^{-1}. In a case of lactose-free milk-based substrate, added concentration remained the same throughout an entire period of cold storage ($N_{end} = 1.1 \times 10^9$ CFU mL^{-1}). The numbers of *L. plantarum* in water-based substrate increased during the first day of cold storage about 2 log orders that remained stable until the end of storage phase and were similar to the numbers of Fresco culture ($N_{end} = 2.7 \times 10^9$ CFU mL^{-1}).

The reduction in pH did not differ a lot among all three examined chocolate substrates. The fastest drop was recorded in case of lactose free milk (1.2 unit) with rate -0.035 h^{-1}. After 21 days of storage period, pH value decreased for another 0.48 units to a final 4.52. The rate of pH drop in milk-based substrate was calculated to -0.298 h^{-1} with a final value 4.54, representing 1.65 unit decrease. In water-based substrate pH decreased about 1.62 unit during fermentation process and the storage period with the reduction rate -0.301 h^{-1}.

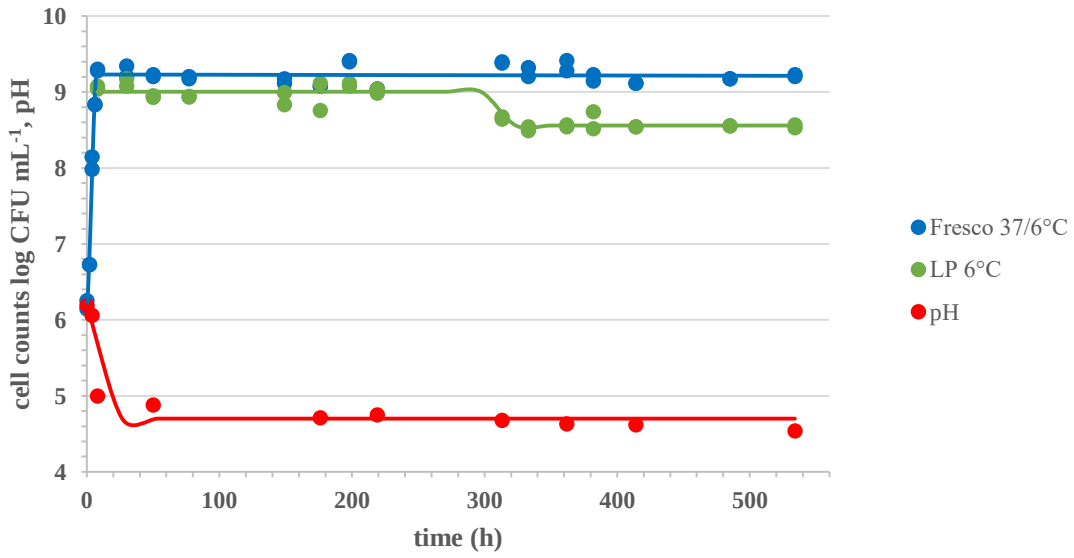

Figure 9. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in milk-based soya substrate with added chocolate during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

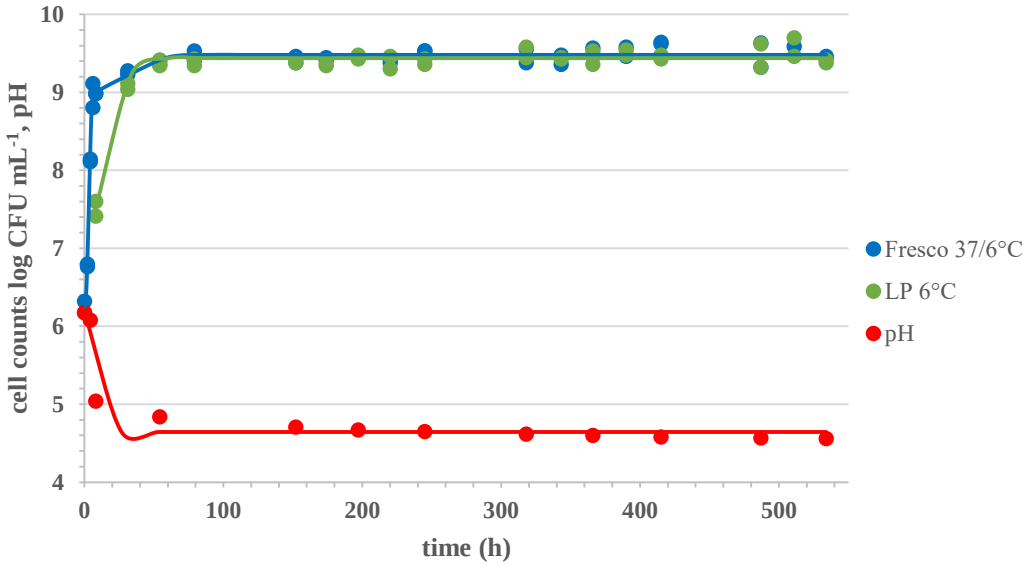

Figure 10. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in water-based soya substrate with added chocolate during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

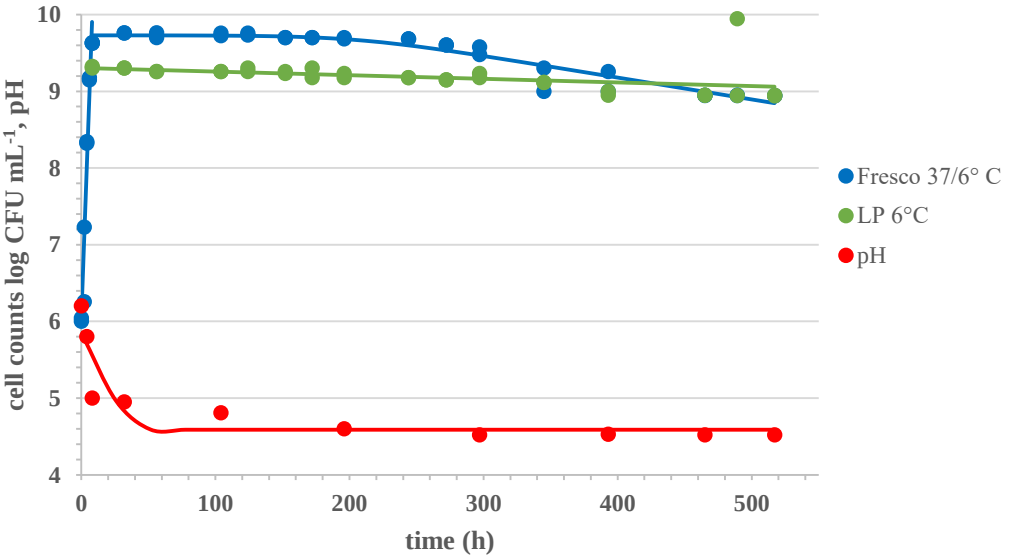

Figure 11. Presumptive counts of the cocci from Fresco DVS 1010 culture and *L. plantarum* (LP) in lactose-free milk soya substrate with added chocolate during fermentation at 37 ± 0.5°C and cold storage at 6 ± 0.5°C

4.2.4. Discussion of the growth parameters of Fresco DVS 1010 culture and *Lactobacillus plantarum*

Results of cell growth of cocci from Fresco DVS 1010 culture during single fermentation process are, for better interpretation, presented in Table 3. After 8 hours of fermentation using mixed Fresco culture, all prepared soya mashes have been shown as a suitable substrate for the growth of selected lactic acid bacteria. Maximal counts of monitored culture reached 10^9 CFU mL^{-1} after 8 h in all examined substrates from initial 10^5 - 10^6 CFU mL^{-1}. Similar results were obtained in a study of Matejčeková et al. (2015), when Fresco starter culture, at the end of 8 h of fermentation, reached counts 10^8 - 10^9 CFU mL^{-1} from initial 10^6 - 10^7 CFU mL^{-1} representing 2-3 log increase. Pelikánová et al. (2015) reported significantly lower concentrations of different starter cultures from the genus *Lactobacillus* during fermentation of water-based amaranth and maize mashes compared to milk-based indicating lower growth and stability of starter cultures in products without the milk component. Despite the higher content of specific nutrients in milk, obtained results show similarity in cell population of Fresco culture in milk, water and lactose-free milk substrates. This could be explained with an adequate nutritional composition of soy flour for the growth of studied Fresco culture. During the cold storage at 6 ± 0.5°C for 21 days, viable counts of cocci from Fresco DVS 1010 were not reduced and were similar to those reached after the fermentation process in all water- and milk-based substrates. On the other hand, in all prepared lactose-free milk substrates a decrease of 1 log order was reported. In general, growth rates of Fresco ranged from 0.416 to 0.714 log CFU mL^{-1} h^{-1}. In a study of Petruláková and Valík (2015), calculated growth rates of *Lactobacillus rhamnosus* GG in a porridge prepared of soy flour were 2-3 times lower (G_r = 0.21 log CFU mL^{-1} h^{-1}), while in other leguminous porridges ranged from 0.2 to 0.35 log CFU mL^{-1} h^{-1}.

In general, the fastest growth was calculated in milk-based substrates, with an exception of the water chocolate substrate wherein the fastest growth rate was observed. In this case, the longest lag phase duration was observed (81 minutes). Other authors also report the fastest growth rate in substrates with the longest lag phase duration (Marko et al., 2014; Kocková et al., 2013). In case of milk substrates with flavouring compounds, short lag phase was observed, while in all others Fresco culture immediately entered an exponential phase of growth. Although Matejčeková et al. (2017; 2015) reported that addition of flavouring compounds in amaranth and buckwheat mashes influenced growth rates of Fresco cultures, no significant differences in soya substrates were observed ($P > 0.05$).

During the fermentation process, organic acids production, as a result of metabolic activity of the cocci of Fresco culture, was observed, that caused the decrease of pH values in all prepared soya substrates. During the fermentation process, a decrease of pH values to 5.0-5.1 from initial 6.0-6.2 was observed (Tab. 3). In comparison, pH values of leguminous porridges fermented with *Lactobacillus rhamnosus* GG slightly decreased (from initial 5.9-6.4 to final 5.6-6.0) (Petruláková and Valík, 2015), while pH values of vegetable substrates fermented by lactic acid bacteria ranged from 3.7 to 4.5 after the fermentation (Němečková et al., 2011). Rathore et al. (2012) achieved pH below 3.5 after 24 h, when fermenting barley and malt media by *L. plantarum*. During the fermentation process of buckwheat mashes the application of 5% starter culture of Fresco resulted in reaching the pH levels of 4.42-5.06 from initial 6.08-6.79 (Matejčeková et al., 2017). Milk-based substrates are reported to be more stable in terms of decreasing pH due to their higher buffering capacity. Lower pH values were observed after 21 days storage in water-based cereal puddings (Helland et al., 2004) and amaranth mashes (Matejčeková et al., 2015) in comparison with milk-based ones.

The pH levels of the final soya products were not dependent on the composition of media, whether it was water, milk or lactose-free milk. Final pH values, after 21 days of storage, ranged from 4.5 to 4.7, which represents a decrease of about 1.5-1.7 units during the fermentation and the storage. In substrates with the addition of flavours, final pH was slightly lower in comparison to those with sucrose only. In their study, Kocková et al. (2013) calculated higher rates of reducing pH in cereal and pseudocereal substrates, where a long lag phase of reducing pH was observed. In case of soya substrates, lag phase of about 4 hours was recorded in almost all, except in water caramel one and both lactose-free milk substrates with the addition of flavours. In those substrates rates of decreasing pH were 10 times lower in comparison with the others (-0.272 h^{-1} to 0.301 h^{-1}).

Table 3. Growth parameters of Fresco DVS 1010 culture, 8 h fermentation at 37 ± 0.5°C in soya substrates

SUBSTRATE soy flour	**Gr (log CFU mL^{-1} h^{-1})**	**λ (h)**	**pH_0**	**pH_{end}**	**k_{pH} (h^{-1})**
milk	0.553	-	6.14	4.70	-0.293
milk + caramel	0.595	0.65	6.14	4.53	-0.272
milk + chocolate	0.561	0.86	6.19	4.54	-0.298
water	0.533	-	6.19	4.61	-0.276
water + caramel	0.416	-	6.03	4.54	-0.021
water + chocolate	0.714	1.35	6.18	4.56	-0.301
lactose-free milk	0.459	-	6.15	4.68	-0.282
lactose-free milk + caramel	0.473	-	6.15	4.50	-0.034
lactose-free milk + chocolate	0.482	-	6.20	4.52	-0.035

(Gr- growth rate, λ- lag phase duration, pH_0- initial pH, pH_{end}- final pH, k_{pH}- rate constant for the decrease of pH)

Although there are no set standards concerning the population of the probiotic organism at the end of the product shelf life, the minimum levels of 10^6 CFU mL^{-1} are usually considered as acceptable, in order to provide health benefits (Georgieva et al., 2009). From the obtained results, it can be seen that the added concentration of *Lactobacillus plantarum* (Tab. 4) remained above the recommended levels during 21 days of cold storage with final counts ranging from 7.70 to 9.44 log CFU mL^{-1}. The different cell population could be attributed to the differences of prepared soy media. While in a study of Matejčeková et al. (2017) the highest counts of *L. rhamnosus* GG in milk caramel product made from buckwheat flour were observed, in case of milk caramel soya substrate the fastest decrease in numbers of *L. plantarum* was observed.

Table 4. Parameters evaluating behaviour of *Lactobacillus plantarum* in fermented soya substrates during storage at 6 ± 0.5°C when added after fermentation

SUBSTRATE soya flour	**k_d (log CFU mL^{-1} h^{-1})**	**stability of counts (h)**	**N_0 (log CFU mL^{-1})**	**N_{end} (log CFU mL^{-1})**
milk	-0.0009	-	9.29	8.83
milk + caramel	-0.0242	475.3	8.93	7.70
milk + chocolate	-0.0431	296.9	9.00	8.56
water	-0.0116	415.6	9.24	7.93
water + caramel	0.0037	-	9.12	9.40
water + chocolate	0.0696	-	7.51	9.44
lactose-free milk	-0.0006	-	9.29	8.96
lactose-free milk + caramel	-0.0008	-	9.34	8.91
lactose-free milk + chocolate	-0.0005	-	9.30	9.06

(k_d- rate constant for the decrease of the *L. plantarum* numbers, N_o- initial counts, N_{end}- final counts)

In amaranth and maize mashes, counts of viable lactobacilli (among them *L. plantarum*) were well maintained above the suggested minimum limit of 10^6 CFU mL^{-1} (Pelikánová et al., 2015) and in oat-based probiotic drink viable cell counts of *L. plantarum* were above 10^9 CFU mL^{-1} (Angelov et al., 2006) during 21 days of storage at 6°C. *L. plantarum* strains maintained also good viability in fermented milk ranging from 6.8 to 7.5 log orders for 28 days during the cold storage (Georgieva et al., 2009). Other probiotic species, like *L. rhamnosus* GG, were able to survive in fermented cereal, pseudocereal, and leguminous substrates throughout 21 days of cold storage at 5°C (Petruláková and Valík, 2015; Kocková and Valík 2014; Kocková et al., 2013).

5. CONCLUSIONS

Based on the presented results and the discussion, the following conclusions are reached:

1. All the prepared soya substrates have proved to be suitable substrates for lactic acid fermentation and growth of starter culture reaching maximal cell counts of 10^9 CFU mL^{-1}. In a cell population of Fresco DVS 1010 culture, no significant differences regarding the addition of flavours in media (milk, water and lactose-free milk) were recorded.
2. Human derived isolate *Lactobacillus plantarum* remained above the recommended levels of 10^6 CFU mL^{-1} (minimum level expected for probiotic strain to achieve health benefits) during an entire storage period of 21 days in all examined soya substrates.
3. In UHT lactose-free milk (1.5% fat), potentially probiotic isolate *L. plantarum* showed good growth properties in the temperature range from 8 to 40°C, with calculated optimal temperature for growth using CTMI model, T_{opt} = 33.7°C. Studied isolate *L. plantarum* reached counts in average 10^7 CFU mL^{-1} at all studied temperatures (except for marginal 8°C) and showed a decrease of pH about 0.17-1.16, in comparison to initial values. All the acquired results, depending on the growth dynamics of studied isolate, are important for the future industrial applications.
4. Taking into account all the above, fermented soya substrates may find its industrial application as a suitable option for the development of new probiotic foods. Further examinations should be focused on a sensory evaluation that would answer the question if they are acceptable to the consumers' demands. Also, intending to study the behaviour of undesirable microbiota in the products developed under this study, to propose an acceptable shelf life represents another important step in the evaluation. However, this should be realised in products from an operational batch, manufactured under real good manufacturing and hygienic practice.

6. REFERENCES

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