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Development of New Products: Probiotics and Probiotic Foods

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Additional information is available at the end of the chapter

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1. Introduction

Probiotics are live microorganisms that confer a beneficial effect on the host when administered in proper amounts [1, 2]. Their beneficial effects on gastrointestinal infections, the reduction of serum cholesterol, the protection of the immune system, anti-cancer properties, antimutagenic action, anti-diarrheal properties, the improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection, Crohn's disease, restoration of the microflora in the stomach and the intestines after antibiotic treatment, etc. are proven by addition of selected strains to food products [3, 4, 5, 6].

Lactobacilli and bifidobacteria are normal components of the healthy human intestinal microflora. They are included in the composition of probiotics and probiotic foods because of their proven health effects on the body [7, 8, 9]. They are the main organisms that maintain the balance of the gastrointestinal microflora [10].

Not all strains of lactobacilli and bifidobacteria can be used as components of probiotics and probiotic foods, but only those that are of human origin, non-pathogenic, resistant to gastric acid, bile and to the antibiotics, administered in medical practice; they should also have the potential to adhere to the gut epithelial tissue and produce antimicrobial substances; they should allow the conduction of technological processes, in which high concentrations of viable cells are obtained as well as to allow industrial cultivation, encapsulation and freeze-drying and they should remain active during storage [11, 12]. This requires the mandatory selection of strains of the genera *Lactobacillus* and *Bifidobacterium* with probiotic properties. Moreover, the concentration of viable cells of microorganisms in the composition of probiotics should exceed 1 million per gram [13] in order for the preparation to exhibit a therapeutic and prophylactic effect.

Along with probiotics probiotic bacteria are most frequently included in the composition of dairy products - yogurt, cheese, etc. [14, 15]. A dairy product that delivers viable cells of

L.acidophilus, *L.bulgaricus*, *Bifidobacterium* sp. is bio-yoghurt. Adequate numbers of viable cells, namely the "therapeutic minimum" need to be consumed regularly for transfer of the "probiotic" effect to consumers. This requires, according to Rybka & Kailasapathy, 1995 [16] the consumption of 100 g per day bio-yoghurt containing more than 10^6 cfu/cm³ viable cells.

The species *L.bulgaricus* is a heterogeneous group of bacteria, including strains with probiotic properties [17]. The inclusion of such cultures in yogurt would transform this lactic acid product into a probiotic product.

Probiotic bacteria are included as components of the starter cultures for non-dairy foods [18]. For each type of non-dairy product strains that can grow in the food environment and contribute to the formation of the sensory profile are selected. So in starter cultures for raw-dried meat products probiotic bacteria that are able to grow in the meat environment are included; in soy fermented foods as components of the starter cultures lactobacilli and bifidobacteria strains which can grow and multiply in soy milk are applied; in fruit and vegetables and fruit and vegetable juices microorganisms with probiotic properties suitable for this type of food are used [19].

Some strains of lactobacilli with probiotic potential are used as components of sourdough in bread-making to extend the shelf life and to improve the quality and some technological properties of the final product [20, 21, 22, 23].

In this chapter, the new steps in obtaining probiotics and probiotic foods are discussed. The requirements for the strains of microorganisms which are implemented as components of the probiotics and probiotic foods are listed.

The chapter includes some data from the research of our research team in the field of selection of bifidobacteria and lactobacilli with probiotic properties, developing the technology for obtaining the probiotics "Enterosan", probiotic milk and beverages, probiotic starter cultures for meat foods and non-traditional fermented probiotic foods.

2. Microorganisms with probiotic properties

Enormous amount of microbial biomass inhabits the stomach and the intestines and accompanies individuals throughout their lives. Organisms that are a part of the gastrointestinal microflora, include saprophytic, pathogenic and conditionally pathogenic microorganisms, enterobacteria, lactobacilli, lactic acid cocci, bifidobacteria. They occupy a niche in the digestive tract and enter into complex relationships both among themselves and with the host - man or animal. Depending on the composition of food intake the diversity of species and the ratio between them varies significantly. Upon intake of plant foods fermenting species predominate, while in meat meal representatives of the putrefactive microorganisms take the upper hand. Microbes transform nutrients in food in different ways and excrete metabolites with diverse chemical nature. Through them the gastrointestinal microflora influences the condition and the health of the body. A part of the microflora that includes lactobacilli and bifidobacteria utilizes the substrates and forms

metabolites as a result of its vital activity through which it oppresses and expels pathogenic and toxigenic bacteria from the biological niche. The degradation of nutrients from the decay performed by pathogenic and toxigenic microorganisms, which include the pathogenic genera *Clostridium* and *Bacteroides* leads to the formation of toxins and products of decay that inhibit the functioning of the organisms and cause diseases. The balance between these two groups of microorganisms determines to a considerable extent the health of the individuals. Many factors affect this balance - the quality of food, water and air, the neuro-psychological status and stress, the social and personal hygiene, the health and the use of drugs, antibiotics, etc. The age of the individuals also influences the diversity of the microflora in the stomach and intestines.

Maintaining the right balance between the species in the gastrointestinal tract is achieved through the adoption of beneficial flora (lactobacilli and bifidobacteria) in the form of concentrates of viable cells, known as probiotics, or in the composition of foods that can be enriched with them.

Probiotics are biologically active preparations containing high concentrations of beneficial natural microorganisms that allow maintaining a predominantly beneficial microflora in the gastro-intestinal tract, ensuring good health and quality of life. In the last decades, science and health care are paying serious attention to probiotics as preventive and therapeutic tools against many diseases. The first beneficial effect of their adoption is the normalization of the gastrointestinal microflora and the occurrence of recovery processes in the digestive tract. This helps to improve the health status of other organs and systems. The practical application of probiotics clearly speaks in favor of this claim. Probiotic microorganisms should be regarded as an indispensable ingredient of food. Absence, lack or destruction of part or all of the useful microflora poses serious hazards to human health. Therefore, one can neither exist without the normal probiotic microorganisms nor can replace them with something else. Neglecting this requirement is associated with serious consequences for the health and life of humans and animals. Quite often probiotics are the only key to the treatment of some diseases of gastroenterological, functional and deficiency nature.

By applying advanced technologies for fermentation, encapsulation and freeze-drying probiotic preparations (Multibionta, Enterogermina, Reuterina, Enterosan, Florastor) with proven prophylactic and healing action in children and adults against colitis, including ulcerative colitis, gastritis, enteritis, ulcerative disease, intestinal infections, disbacteriosis and some cases of dyspepsia, have been created.

Not all species and strains of lactobacilli and bifidobacteria could act as regulators of the gastrointestinal microflora, but only those who are able to survive and grow under the different conditions of the digestive tract. This requires the selection of strains of lactobacilli and bifidobacteria with probiotic properties, which are reflected in their ability:

1. To be part of the natural microflora in humans and animals.
2. To have the ability to adhere to epithelial cells or cell lines, or at least to be able to colonize the ileum temporarily [24, 25].

Adhesion can be nonspecific - related to the physicochemical factors, and specific - based on specific molecules on the surface of the probiotic cells that adhere to receptor molecules on the surface of the epithelial cells. The strains used in the production of fermented milk products are not with the best adhesion properties, while probiotic bacteria show strong adhesion that is species specific. As far as their ability to adhere is concerned lactic acid bacteria (including lactic acid bacteria used in the manufacture of milk products) show moderate to good adhesion properties when it comes to adhesion on human cell lines [26, 27, 28].

The adhesion of probiotic strains to the surface of the intestine and the subsequent colonization of the gastrointestinal tract of humans creates conditions for better retention in the intestinal tract and implementation of metabolic processes with a strong immunomodulatory effect. Adhesion provides interaction with the mucosa, supporting the contact with the intestine-associated lymphoid tissue, which in turn provides stabilization of the intestinal mucosa that performs a barrier function. The intestine-associated lymphoid tissue can interact with the cells of the probiotic strains and their components and thus has a positive effect on the immune system of the host [29].

In many species of lactic acid bacteria, including those of the genus *Lactobacillus*, the presence of surface-layer proteins [30, 31, 32] has been found. The gene for the S-layer protein has been sequenced and cloned in *Lactobacillus brevis* [33], *Lactobacillus acidophilus* [34], *Lactobacillus helveticus* [35] and *Lactobacillus crispatus* [36].

The thickness of the surface-layer (S-layer) in bacteria is typically 5 to 25 nm and it is composed of subunits arranged in a grid (lattice) with irregular, square or hexagonal symmetry [37]. In the amino acid analysis of the S-layer proteins it was found that they are rich in acidic and hydrophobic amino acids and very poor in sulfur-containing amino acids [38]. In determining the secondary structure it was found that in most S-layer proteins 40% of the amino acids form a β -sheet structure and 10-30% - α -helix [38]. Common feature of all surface-layer proteins characterized so far is their ability to crystallize spontaneously into a two-dimensional layer on the outer side of the bacterial cell wall.

In representatives of the genus *Lactobacillus* some surface located enzymes are established along with the S-layer proteins on the cell surface. The molecular weight of the S-layer proteins in lactobacilli ranges from 40 kDa to 60 kDa and they are one of the smallest known S-layer proteins [31, 36, 39, 40]. Compared with many other S-layer proteins, which are of acidic nature, those in lactobacilli are characterized by high values of their isoelectrical points [41]. The S-layer proteins in some lactobacilli give the cell surface hydrophobicity [40, 42]. Moreover, the hydrophobicity of the cell surface of the strain *Lactobacillus acidophilus* ATCC 4356 can be varied in accordance with the change of the ionic strength of the medium [43]. In a sequencing study of the S-layer proteins in *Lactobacillus acidophilus*, *Lactobacillus crispatus* and *Lactobacillus helveticus* a high degree of homology in one third of their C-terminus is demonstrated [36].

The functions of the S-layer proteins in lactobacilli are insufficiently studied. The S-layer proteins act as adhesins in many bacteria such as lactobacilli and some representatives of the genus *Bacillus*, so they determine their adhesion to epithelial cells or extracellular matrix proteins [44, 45, 46, 47].

3. To survive in the conditions of the stomach and intestines, i.e. to survive in the conditions of acidic pH in the stomach and to withstand the effects of bile [48, 49, 50].

The survival of bacteria in gastric juice depends on their ability to tolerate the low pH values of the medium. Transition time in these conditions depends on the condition of the individual and the type of the food and it ranges from 1 to 3-4 hours. The lactic acid bacteria *L.sakei*, *L.plantarum*, *L.pentosus*, *P.acidilactici* and *Pediococcus pentosaceus* can survive in acidic conditions [51, 52]. Therefore Klingberg et al., 2006 [51] and Pennacchia et al., 2004 [52] suggest the examination of the survival of the strains for probiotic purposes in cultural medium at pH 2.5, acidified with hydrochloric acid for 4 h. Using this criterion *Lactobacillus* strains resistant to low values of pH (pH 2) and the presence of pepsin [17] are selected.

Bacteria that survive in the conditions of the stomach then enter the duodenum, where bile salts are poured and their concentration is 0.3%. Microorganisms reduce the emulsifiable effect of bile salts by hydrolyzing them, thus reducing their solubility. Some intestinal lactobacilli, such as *L.acidophilus*, *L.casei* and *L.plantarum* have the ability to hydrolyze bile salts [53]. Moreover, some strains of lactic acid bacteria isolated from sausages such as *L.sakei*, *L.plantarum*, *L.pentosus* and *P.acidilactici* are resistant to 0.3% bile salts [51, 52].

The survival of probiotic bacteria in the gastrointestinal tract, their translocational and colonizational properties and the destruction of their active components are essential for the realization of their preventive role.

Different probiotic strains react differently in different parts of the gastrointestinal tract - some strains are killed very quickly in the stomach, while others pass through the entire gastrointestinal tract, retaining high concentrations of viable cells [29, 54, 55, 56, 57, 58, 59, 60].

The natural gastro-intestinal microflora, especially lactobacilli, should have the ability to hydrolyze conjugated bile acids that are present in large quantities in the intestines. Conjugated bile acids provide the emulsification, digestion and absorption of lipids more efficiently than bile acids in non-conjugated form. The hydrolysis of bile acids may be associated with the accumulation of energy in anaerobic conditions and / or the detoxication of bile acids that inhibit bacterial growth.

4. To have the ability to reproduce in the gastrointestinal tract. By primarily utilizing the substrate to oppress and expel from the biological niche the pathogenic and toxigenic microorganisms.

5. To possess antimicrobial activity against conditionally pathogenic, carcinogenic and pathogenic microorganisms, which is associated with inactivation of their enzyme systems, overcoming their adhesion, growth suppression and forcing them out of their biological niche, as a result of which gastrointestinal microflora is normalized.
6. To produce antimicrobial substances.

Probiotic strains should be able to carry out fermentation with lactic acid and bacteriocin production by utilizing the carbohydrates, thus changing the pH of the medium and suppressing the development of pathogenic and toxigenic microorganisms or acting directly on the microbial cells by producing antibacterial substances with peptide nature (bacteriocins) [61, 62].

7. To modulate the immune response.
8. To be safe for clinical and food applications.

Lactic acid bacteria applied in clinical and functional foods must be safe, especially if intended for humans.

9. To allow industrial cultivation, resulting in obtaining concentrates with high concentrations of viable cells that can be included in gel matrices (encapsulation), thus retaining their activity in the process of freeze-drying as well as in the composition of the finished products.

Donald and Brow, 1993 [13] and Wolfson, 1999 [63] conclude that in order to prevail in the balance of gastro-intestinal microbial association the number of live beneficial probiotic bacteria should exceed 10^9 per gram product. Achieving this value requires a better understanding of the factors of cultivation, concentration, drying and storage.

3. Lactobacilli and bifidobacteria with probiotic properties – Foundation for the probiotics "Enterosan"

Lactobacilli, bifidobacteria and lactic acid cocci are isolated from different sources (from the intestinal tract of infants naturally fermented raw-meat dried products, naturally fermented sourdough, fermented vegetables, etc.) by contemporary breeding and genetic methods, they are identified using the methods of conventional taxonomy (morphological, physiological, biochemical, cultural) and molecular genetic methods (ARDRA, pulse gel electrophoresis, RAPD).

As a result of extensive breeding work on a wide range of strains of lactobacilli and bifidobacteria, strains suitable for incorporation in starter cultures for fermented milk products, probiotics and probiotic foods and beverages that have the ability to reproduce in the model conditions of digestion, to synthesize lactic and other organic acids, bacteriocins, by inhibiting the growth of pathogens that cause toxicity, toxicoinfections and fungal infections are selected. They allow the accumulation of high concentrations of viable cells in the process of fermentation, immobilisation, freeze-drying that retain their viability in storage conditions (Table 1) [64].

Genus <i>Lactobacillus</i>	genus <i>Bifidobacterium</i>	Lactic acid cocci
<i>L.bulgaricus</i> BG <i>L.bulgaricus</i> GB <i>L.bulgaricus</i> BB <i>L.helveticus</i> H <i>L.plantarum</i> 226-15 <i>L.plantarum</i> Sw <i>L.casei</i> C <i>L.acidophilus</i> 2 <i>L.acidophilus</i> A	<i>B.bifidum</i> 1H <i>B.bifidum</i> L1 <i>B.infantis</i> <i>B.breve</i> <i>B.longum</i>	<i>Pediococcus pentosaceus</i> <i>Lactococcus lactis</i> L4 <i>Streptococcus thermophilus</i> T3

Table 1. Strains of lactobacilli, lactic acid cocci and bifidobacteria with probiotic properties

The human organism is a complex biological system, which requires nutrients, air, water and energy for performing the thousands of biochemical reactions, which provide its normal functioning. The food in the stomach is subjected to transformation under the action of enzyme systems and with the direct participation of microorganisms. A part of them, which are related to the genera *Lactobacillus* and *Bifidobacterium*, form the group of the beneficial microorganisms. They digest substrates and through the metabolites, produced as a result of their vital activity, they inhibit and expel from the biological niche the pathogenic, toxigenic and putrefactive microorganisms.

The assimilation of nutrients by the toxigenic and putrefactive microorganisms, which form the group of the undesired microflora, leads to the synthesis of putrefactive and toxic metabolites, which impede the functions of separate systems and the organism as a whole.

Pathogenic microorganisms enter the digestive tract of humans and animals and cause digestive disorders and inflammation of the intestinal mucosa, when present in high concentrations (above 10^5 cfu/g).

Some of the metabolites produced by lactic acid bacteria and bifidobacteria are lactic, acetic, citric and other organic acids, through which they acidify the medium and inhibit the growth of pathogens. Another group of substances with antimicrobial action are bacteriocins, which have protein nature.

The interactions between the selected group of lactobacilli [17] and bifidobacteria and the pathogens, representatives of *Enterobacteriaceae*, causing toxicoinfections and toxicoses, as well as fungal pathogens and the cancerogenic *Helicobacter pylori* are of great interest.

Pathogenic microorganisms of human origin - *Salmonella* sp., *Candida albicans*, *Proteus vulgaris*, *Enterococcus faecalis*, *Staphylococcus aureus* subsp. *aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* subsp. *pneumoniae*, *Escherichia coli* with viable cell counts of the suspensions above 10^{10} cfu/cm³ are used as test-microorganisms. The investigations are conducted using the agar diffusion method. The results from these experiments are presented in Table 2.

Bifidobacteria have inhibitory activities close to that of lactobacilli (Table 2). When cultivated together, *B. breve*, *B. infantis*, *B. longum* and *B. bifidum* L1 exhibit greater antimicrobial effect in comparison with each one of the strains separately. The titratable acidity of the liquid supernatant is comparatively higher as well. They demonstrate certain synergism, which has a positive effect on human and animal organisms. Having in mind their distribution in the gastro-intestinal tract, they are the main regulators of the microflora in the colon.

Bifidobacteria belong to the symbionts particularly important to the human and animal organism. They are some of the first inhabitants of the digestive tract of the new-born mammals. Their importance is strengthened by their regulatory role in the colon.

Bifidobacteria have active metabolism, producing other organic acids (acetic, citric, tartaric) beside lactic acid. They exhibit antimicrobial activity against pathogenic and toxigenic microorganisms. Their significant synergism with lactobacilli and the rest of their probiotic properties, as well as their important place of habitat, define the important health-promoting role of bifidobacteria.

Strain Test- microorganism	<i>Bif.breve</i>	<i>Bif.longum</i>	<i>Bif.infantis</i>	<i>Bif.bifidum</i> L1	Bifidobacterium symbiotic culture
<i>Salmonella</i> sp., 1,2.10 ¹² cfu/cm ³ *	14	9	7	10	15
<i>C. albicans</i> , 5.10 ⁸ cfu/cm ³	10	9 – 10	10	10	10
<i>P.vulgaris</i> , 5.10 ¹¹ cfu/cm ³	11	9	7	8	13
<i>E. faecalis</i> , 2,2.10 ¹¹ cfu/cm ³	13	11	10	9	12 – 13
<i>S. aureus</i> , 1,0.10 ¹² cfu/cm ³	8	10	10	-	8
<i>P.aeruginosa</i> , 7.10 ¹⁰ cfu/cm ³	11	7	8	8	10
<i>K. pneumoniae</i> , 1,0.10 ¹¹ cfu/cm ³	20	18	19	21	20 – 21
<i>E.coli</i> 1,5.10 ¹⁰ cfu/cm ³	12	10	9	11	10 – 11

* concentration of the cells of the test-microorganism in the agar medium.

Table 2. Antimicrobial properties of bifidobacteria

The antimicrobial activity of *L. acidophilus* 2, *L. bulgaricus* NBIMCC 3607, the symbiotic culture of *B. bifidum* L1, *B. longum*, *B. breve* on the growth of 11 strains of *H. pylori* of human origin is determined.

The symbiotic culture of bifidobacteria demonstrates the highest inhibitory effect on *H. Pylori* – the zones of inhibition are >10 mm for 50% of the strains (Table 3.)

L. acidophilus 2 and *L. bulgaricus* GB suppress the growth of half of the investigated strains of *Helicobacter pylori* (Table 3). It must be noted, that the model investigations on the influence of the tested cultures on the cells of *H. pylori* are conducted with liquid concentrates of *L. acidophilus* 2, *L. bulgaricus* GB, symbiotic culture of *B. bifidum* 1, *B. longum*, *B. breve* with viable cell counts above 10^{10} cfu/cm³ and pH of the fermentation medium 6,3. This means that the action of part of the metabolites with antimicrobial activity is eliminated.

<i>H. pylori</i> Mc Farland	Inhibition zone of <i>H. pylori</i> , mm			pH
	<i>L. acidophilus</i> 2	<i>L. bulgaricus</i> GB	Symbiotic culture of bifidobacteria	
MF=1	7(10)	7(17)	7(12)	6,3
MF=0.5	7	7	7	6,3
MF =0.5	7	7	7	6,3
MF =0.5	7,5	7	7	6,3
MF=0,5	7	7	15,5	6,3
MF =0.5	9,3	10	13	6,3
MF=0.5	26	20	12	6,3
MF=0.5	7	7	20	6,3
MF=2	9	11	10,2	6,3
MF=0.5	11	9,7	14	6,3
MF=0.5	7	7	8,5	6,3

Table 3. Antimicrobial activity of *L. acidophilus* 2, *L. bulgaricus* GB, the symbiotic culture of *Bif. bifidum* 1, *Bif. longum*, *Bif. breve* against *H.pylori*

4. Antibiotic resistance of bifidobacteria

Antibiotics are substances with antimicrobial action, which influence both Gram-positive and Gram-negative bacteria. They inhibit the growth of or destroy microbial cells. In order to fulfill these functions, the antimicrobial substances must penetrate the cell, conjugate with a certain cell structure, which participates in a vital processes (DNA replication and cell division) or suppress them completely.

The effect of 22 antibiotics - β -lactam (penicillin, ampicillin, cefamndole, ciprofloxacin, amoxicillin, oxacillin, piperacillin, azlocillin), aminoglicoside (streptomycin, gentamicin, kanamycin, lincomycin, clindamycin, amikacin, vancomycin, tobramycin), macrolide (rifampin, erythromycin), tetracycline (tetracycline, doxycycline), aromatic (chloramphenicol)

and nalidixic acid, on the growth of the selected lactobacilli - is studied. The 22 antibiotics belong to 3 groups with different mechanism of action – inhibition of the synthesis of the cell walls (penicillin, ampicillin, cefamndole, amoxicillin, oxacillin, piperacillin, azlocillin, vancomycin), inhibition of the protein synthesis (streptomycin, gentamicin, kanamycin, lincomycin, clindamycin, amykacin, tobramycin, rifampin, erythromycin, tetracycline, doxycycline, chloramphenicol), inhibition of the synthesis of DNA and/or cell division (ciprofloxacin and nalidixic acid). The investigated concentrations are equivalent to the actual concentration in *in vivo* antibiotic therapy.

All four strains of bifidobacteria (*Bif.bifidum* L1, *Bif.breve*, *Bif.infantis*, *Bif.longum*) are resistant to the action of most of the studied antibiotics with *Bif.bifidum* expressing the best results, followed by *Bif.breve*, *Bif.infantis* and *Bif.longum*. They show some sensitivity towards the action of aminoglycoside antibiotics. *Bif.bifidum* L1 demonstrates dense growth when tested against 18 out of the 22 antibiotics, weak growth when examined against 3 of the 22 antibiotics and it has single colonies in the clearance zone when tested against vancomycin. *Bif.breve* shows the following results: dense growth - 9 out of 22 antibiotics, weak growth – 11 out of 22 antibiotics, no growth – 2 out of 22 antibiotics. *Bif.infantis* exhibits dense growth when tested against 5 out of 22 antibiotics, weak growth – 12 out of 22 antibiotics, single colonies in the clearance zone – 3 out of 22 antibiotics, no growth – 2 out of 22 antibiotics. *Bif.longum* is characterized with dense growth when examined against 15 out of 22 antibiotics, weak growth – 6 out of 22 antibiotics, no growth – 1 out of 22 antibiotics. These results reveal the possibility for the inclusion of the strains in the complex therapy against different diseases.

The resistance of the cells of the different *Lactobacillus* [17] and *Bifidobacterium* strains to 22 of the most frequently applied in medical treatment antibiotics reveals the possibility for their application in the cases of disbacteriosis. Moreover, it is better to use strains with natural polyvalent resistance as components of probiotics for the treatment of disbacteriosis.

5. Survival of bifidobacteria in the model conditions of the digestive tract

Bifidobacteria survive in the model conditions of the digestive tract – at low pH values in the presence of enzymes (pH=2 + pepsin) and at neutral pH values in the presence of enzymes (pH=7 + pepsin) (Fig. 1). The cells of the four strains are more sensitive to pH=2 + pepsin than to pH=7 + pepsin. At pH=2 + pepsin a reduction in the number of viable cells is observed; the reduction is by over 2 to approximately 5 log cfu/g at the 24th hour from the beginning of the cultivation in comparison to the baseline concentration of viable cells in the population; *Bif.infantis* and *Bif.longum* are more sensitive to pH=2 + pepsin than *Bif.breve* and *Bif.bifidum* L1. At pH=7 + pepsin the reduction in the number of viable cells is by over 1 to approximately 3 log cfu/g at the 24th hour from the beginning of the cultivation in comparison to the baseline concentration of viable cells in the population; *Bif.infantis* and *Bif.longum* are more resistant to pH=7 + pepsin than *Bif.breve* and *Bif.bifidum* L1.

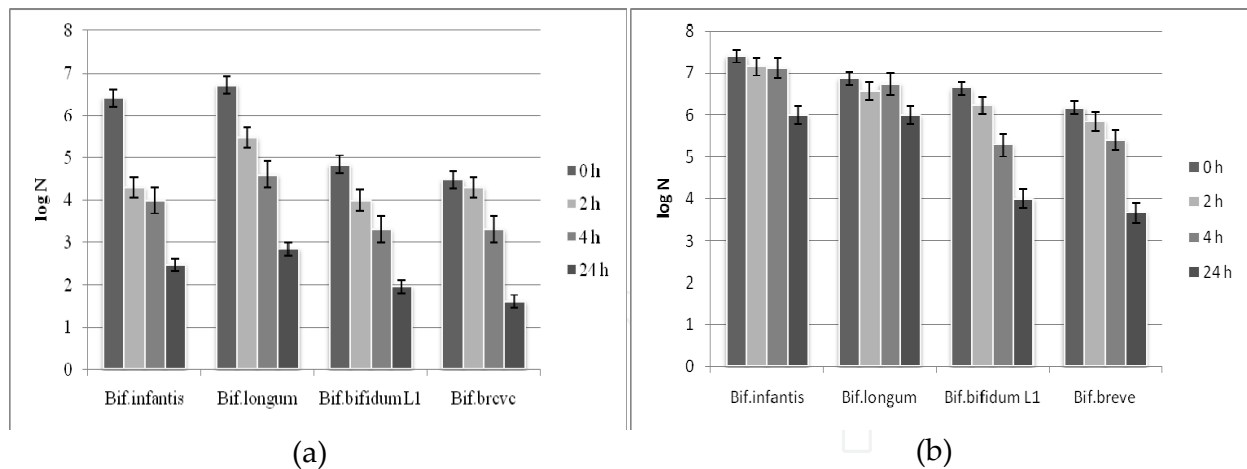


Figure 1. Reduction of the viable cells of bifidobacteria at pH=2 + pepsin (a) and at pH=7 + pepsin (b)

All bifidobacteria strains tested for their resistance to different concentrations of bile salts maintain high levels of viable cells (Fig. 2). An increase in the titre of viable cells at 0,15% bile salts is observed in *Bif. infantis* (Fig. 2a), *Bif. bifidum* L1 (Fig. 2b) and *Bif. longum* (Fig. 2c), while in *Bif. breve* (Fig. 2d) the number of viable cells at 0,15% bile salts decreases from the very beginning of the experiment. At 0,3% bile salts the number of viable cells of *Bif. bifidum* L1 (Fig. 2b) and *Bif. longum* (Fig. 2c) increases during the first 8 hours, but at the 24th hour the cell count is lower than the value at the 8th hour in both the two strains. In *Bif. infantis* (Fig. 2a) and *Bif. breve* (Fig. 2d), the concentration of viable cells starts decreasing from the beginning of the test.

On the basis of these investigations four groups of probiotics „Enterosan” are developed: probiotics for the gastro-intestinal tract, probiotics for promotion of the functions of some endocrine glands, probiotics for functional usage and probiotics for deficiency diseases [65]. They have high concentration of viable cells of probiotic bacteria (over 10^9 cfu/g).

The probiotics "Enterosan" have been tested by leading experts in clinics in our country and abroad and are proven to be beneficial to the human organism - for gastrointestinal infections, rotavirus infections, disbacteriosis due to antibiotics, in chemotherapy, in osteoporosis, arthritis, multiple sclerosis, allergies, anemia, high blood pressure, etc.

The road to developing a probiotic preparation is quite long. It begins with the selection of strains of microorganisms with probiotic properties, the development of probiotic formulations and the implementation of industrial process.

There are several probiotic products on the market but the documentation is often based upon case reports, animal studies or uncontrolled small clinical trials, and only few products declare the content of microorganisms [66].

In the conducted studies on the probiotic properties of different species and strains differences not only between different types of probiotic bacteria, but also between strains within a species are established; differences that should be taken into account in the selection of strains with probiotic properties for industrial use.

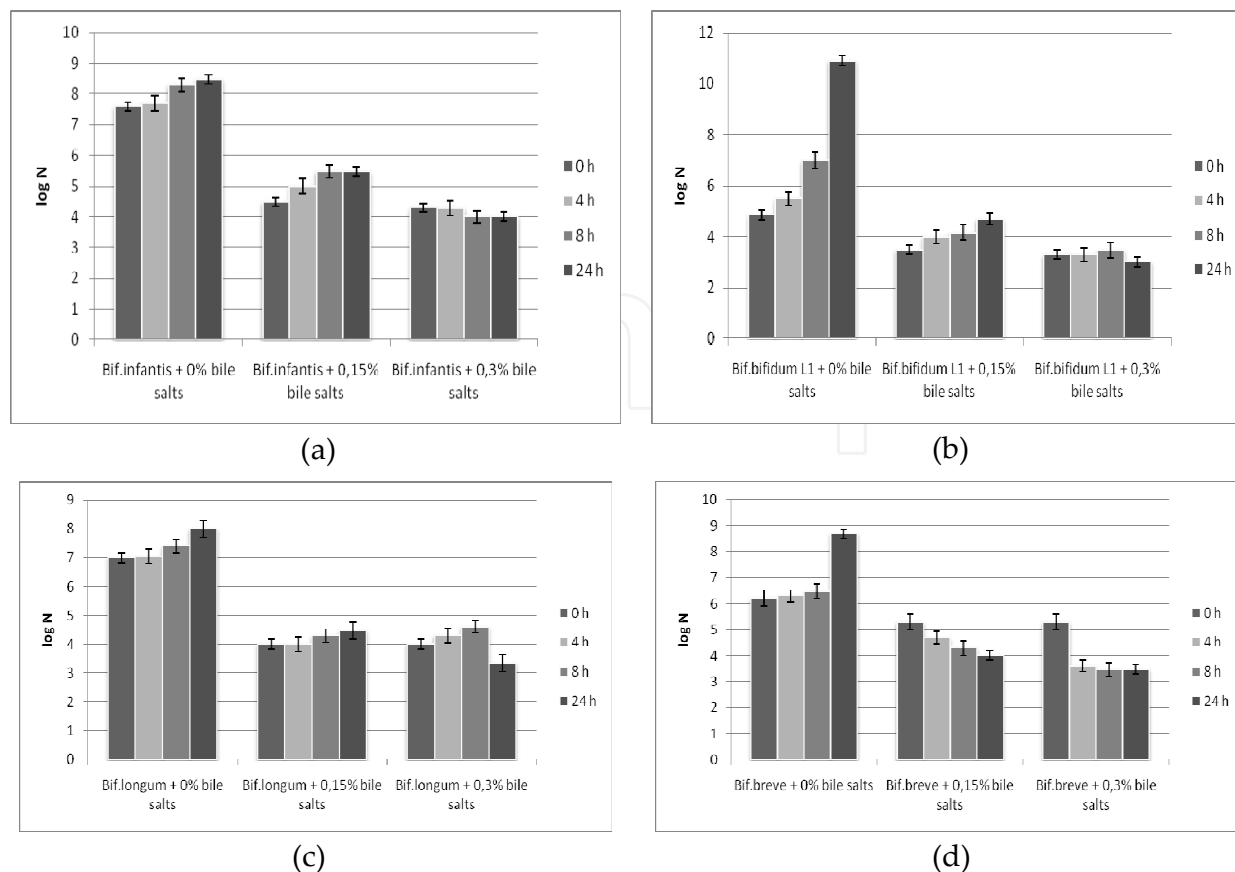


Figure 2. Change in the concentration of viable cells of *Bif. infantis* (a), *Bif. longum* (b), *Bif. bifidum* L1 (c), *Bif. breve* (d) at different concentrations of bile salts

6. Probiotic foods

6.1. Yoghurt with high concentration of viable cells of the probiotic strain

Lactobacillus delbrueckii subsp. *bulgaricus* NBIMCC 3607

Lactic acid foods occupy a major place in the diet of our contemporaries. About 80% of the population use yoghurt for direct consumption or as a food supplement daily. A characteristic feature of this product is the addition of starters of pure cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* for conducting lactic acid fermentation. By using an appropriate technological process a product with characteristic taste and aroma, physicochemical and biological properties is obtained from milk as a raw material. These traditional lactic acid bacteria have a positive effect on the body, which is a result of the formed metabolites, which inhibit the putrefactive and pathogenic flora or of the improvement of the utilization of lactose [67].

Many functional foods include lactobacilli in their composition (Table 4). Lactobacilli are particularly important in the manufacture of probiotic foods [68]. Several species of the genus *Lactobacillus* are used as starters in the manufacture of yoghurt, cheese and other fermented liquid products [69, 70]. It should be noted that the properties of the strain itself

are particularly important in the selection of probiotic cultures. Not all strains can be cultivated on an industrial scale because of the low reproductive capacity in the medium or because of their low survival rate in the processes of freezing and freeze-drying [71]. That is why the cultures used in the production of fermented foods must meet certain requirements (Table 5).

GENUS	SPECIES
<i>Lactobacillus</i>	<i>L. acidophilus</i> ; <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ; <i>L. casei</i> ; <i>L. crispatus</i> ; <i>L. johnsonii</i> ; <i>L. lactis</i> ; <i>L. paracasei</i> ; <i>L. fermentum</i> ; <i>L. plantarum</i> ; <i>L. rhamnosus</i> ; <i>L. reuteri</i> ; <i>L. salivarius</i> .
<i>Bifidobacterium</i>	<i>B. adolescentis</i> ; <i>B. bifidum</i> ; <i>B. breve</i> ; <i>B. essensis</i> ; <i>B. infantis</i> ; <i>B. lactis</i> ; <i>B. longum</i>
<i>Enterococcus</i>	<i>E. faecalis</i> ; <i>E. Faecium</i>
<i>Pediococcus</i>	<i>P. acidilactici</i>
<i>Propionibacterium</i>	<i>P. freudenreichii</i>
<i>Saccharomyces</i>	<i>S. boulardii</i>
<i>Streptococcus</i>	<i>S. thermophilus</i>

Table 4. Probiotic strains used in the production of fermented milk [72, 73, 74]

The selection of probiotic strains is based on microbiological criteria for food safety of the final product. This is achieved by applying non-pathogenic strains with clear health effects and proper hygiene [75].

The high concentration of viable cells and the good survival when passing through the stomach allow lactobacilli and bifidobacteria in fermented milk products to fulfill their biological role in the intestine.

Several properties of bacteria such as oxygen sensitivity, storage stability, resistance to the proteases of the digestive system, sensitivity to aldehyde or phenolic compounds produced by the metabolism of amino acids, antioxidant activity, adhesion to the intestinal mucosa are examined in *in vitro* testings [88,89]. Strains exhibit the specific properties of lactic acid bacteria in a different degree. The combination of strains with different properties allows the increase in the biological activity of fermented foods. This in turn is related to their ability to develop as symbiotic cultures.

Fermented milk products with probiotic properties are designed on the basis of the experience in the field of development of probiotics. Given that yogurt is the most popular food after bread a technology that includes the use of a starter culture with the probiotic strain *Lactobacillus delbrueckii* subsp. *bulgaricus* NBIMCC 3607, which has high reproductive capacity and meets all the requirements for probiotic cultures, has been developed. The technology is piloted for a period of over 1 year in industry. Table 6 presents the change of the acidity and the concentration of viable cells in the finished product during storage.

Industrial field	Criteria	Product	References
Suppliers of probiotic cultures	Cheap cultivation	Cultures for all groups of products	Charteris et al., 1998 [76]
	Easy concentration for obtaining high cellular density	Cultures for all groups of products	Charteris et al., 1998 [76]
Production of probiotic foods	Possibility for industrial production	Products, produced in high quantities (cheese)	Gomes and Malcata, 1999 [77]
	Compatibility with other lactic acid bacteria	All fermented products	Samona and Robinson, 1994 [78] Nighswonger et al., 1996 [79]
	Stability during storage at acidic conditions	Acidophilous milk, yoghurt, cheese	Micanel et al. 1997 [80] Gobbetti et al., 1998 [81]
	Stability during storage in non-fermented milk	Sweetened acidophilous milk	Brashears and Gilliland, 1995 [82]
	Resistance to bacteriophages	All fermented products	Richardson, 1996 [83]
	Survival in the conditions during the maturation and freezing of the ice cream	Ice-cream	Christiansen et al., 1996 [84]
	Tolerance to preservatives	Non-sterilized products	Charteris et al., 1998 [76]
	Stability during storage at temperatures under -20°C	Ice-cream, frozen products	Modler et al., 1990 [85] Christiansen et al., 1996 [84]
	Tolerance towards oxygen during growth	All fermented products	Gomes and Malcata, 1999 [77]
	Low activity at temperatures under 15°C	Cultures for all groups of products	Gomes and Malcata, 1999 [77]
	Utilization of pentanal and n-hexanal	Soy products	Scalabrini et al., 1998 [86] Murti et al., 1993 [87]
	Fermentation of raffinose and stachyose	Soy products	Scalabrini et al., 1998 [86]

Table 5. Some criteria applied in the selection of probiotic strains for fermented foods

Day	Titrable acidity, °T	Concentration of viable cells, cfu/cm ³		Proportion <i>Str.thermophilus</i> : <i>L.bulgaricus</i>	Extraneous microflora
		<i>Str.thermophilus</i>	<i>L.bulgaricus</i>		
1	104	5x10 ¹¹	5x10 ¹¹	1:1	Not found
15	106	6,5x10 ¹¹	6,45x10 ¹¹	1:1	Not found
30	108	6x10 ¹¹	6x10 ¹¹	1:1	Not found

Table 6. Physicochemical and microbiological indicators of yogurt produced using the new technology

The data show that the yoghurt produced according to this technology lasts for one month, during which the acidity is maintained within the standard requirements and the concentration of viable cells of *L.bulgaricus* NBIMCC 3607 in 1 gram of the product exceeds 1 billion by the end of the prolonged storage. Furthermore, the ratio of streptococci to lactobacilli is within the range of 1:1. A similar result can be achieved in any of the currently used technologies.

High concentrations of lactobacilli in yogurt increase its healing and preventive properties. Thus, the most popular product becomes probiotic.

6.2. Bio-yoghurt

Most of the strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* do not retain in the intestinal tract, which limits the application of yogurt during antibiotic therapy and for other medical purposes. Therefore, probiotic bacteria are included in the composition of starter cultures for lactic acid products in addition to the traditional microorganisms *L.bulgaricus* and *Str.thermophilus*, which turns them into products with medicinal properties, known as bio-yoghurt (yogurt, dry mixes, ice cream, soft and hard cheeses, products for infant feeding).

The microflora of bio-yoghurt includes mainly *L.acidophilus*, *L.paracasei* ssp. *paracasei*, *L.paracasei* biovar *shirota*, *L.rhamnosus*, *L.reuteri*, *L.gasseri*, *Bifidobacterium infantis*, *Bif.breve*, *Bif.longum*, *Bif. bifidum*, *Bif.adolescentis* and *Bif.lactis* [90]. In addition to these species, some products contain *Bif.animalis*, which multiplies faster than other bifidobacteria, but unlike them it is not isolated from the intestinal tract of humans, although some *in vitro* studies show that some strains of *Bif.animalis* have the ability to attach to epithelial cells.

Many researchers believe that only species and strains isolated from the gastrointestinal tract of humans, provide probiotic effects on the human body. The digestive system of the fetus in the womb is sterile. It is inhabited within the first 2-3 days after birth. So right after birth the digestive system is inhabited by species and strains that form its gastro-intestinal microflora, as a result of natural selection, and they are better adapted to the conditions of the gastro-intestinal tract. Through this type of functional foods probiotic bacteria enter the body in the form of fermented milk.

Probiotic lactobacilli attach to special receptors on the epithelial wall and fill the vacant spots in the intestine. They utilize nutrients and produce lactic acid and substances with antimicrobial activity [90]. Their prophylactic role consists in changing the conditions, making them unsuitable for the development of bacteria that cause infections such as *Salmonella* sp. [90]. It has been shown that lactobacilli increase the levels of immunoglobulin Ig A and Ig G [91], thus protecting the immune system, lower cholesterol levels [59, 92], etc.

Bifidobacteria are located on the surface of the colon. In this part of the gastrointestinal tract different types of bifidobacteria utilize nutrients and produce lactic and acetic acids and antimicrobial substances (bacteriocins). The large amount of viable cells of bifidobacteria stimulate the walls of the colon to excrete the polysaccharide mucin that facilitates the passage of faeces through the colon, thereby preventing the colonization of cells of *E.coli*, *Candida* sp. thus protecting the body.

In recent years some yoghurt products have been reformulated to include live cells of strains of *L.acidophilus* and species of *Bifidobacterium* (known as AB-cultures) in addition to the conventional yoghurt organisms, *Str.thermophilus* and *L.bulgaricus*. Therefore bio-yoghurt is yoghurt that contains live probiotic microorganisms, the presence of which may give rise to claimed beneficial health effects [93]. In order to exert its probiotic effect, the number of viable cells of probiotic bacteria in bio-yoghurt should exceed 1 million [94] (10^8 - 10^9 cfu/g) [10]. According to a Japanese standard the number of bifidobacteria in fresh milk must be at least 10^7 viable cells/ml. As far as the National Yoghurt Association (NYA) in the U.S. is concerned in the production of bio-yoghurt the concentration of lactic acid bacteria in the finished products must be 10^8 viable cells of lactic acid bacteria / g. Moreover, the culture must have rapid growth during fermentation as well as acid tolerance in order to maintain high microbial content during storage.

Technologies for obtaining probiotic yogurt from whole milk and lactic acid beverage with bifidobacteria from skimmed cow's milk with the participation of *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and strains of the genus *Bifidobacterium* have been developed. The microbiological indicators of this probiotic milk are presented in Table 7.

With the inclusion of bifidobacteria in the starter culture for yoghurt a product with high concentration of active cells (more than 10^8 cfu/g) with durability of 30 days is obtained (Table 7).

Day	Titrable acidity, °T	Concentration of viable cells, cfu/cm ³			Proportion <i>Str.thermophilus</i> : <i>L.bulgaricus</i>	Extraneous microflora
		<i>Str.thermophilus</i>	<i>L.bulgaricus</i>	Bifidobacteria		
1	114	7×10^{10}	$3,5 \times 10^{10}$	7×10^9	1:2	Not found
15	120	$7,7 \times 10^{10}$	$3,75 \times 10^{10}$	$2,9 \times 10^9$	1:2	Not found
30	126	6×10^9	3×10^9	5×10^8	1:2	Not found

Table 7. Physicochemical and microbiological indicators of the probiotic yogurt with bifidobacteria during storage at $4 \pm 2^\circ\text{C}$

A technology for obtaining fermented milk beverage with bifidobacteria has been developed and implemented. The concentration of viable cells in the product is over 10^9 cfu/cm³, which is consistent with the requirements for the concentration of viable probiotic cells in bio-yogurt, required to perform health beneficial effects. The beverage retains the concentration of bifidobacteria cells for 40 days when stored at $4 \pm 2^\circ\text{C}$ (Table 8).

Day	Concentration of viable cells, cfu/cm ³		Titrable acidity, °T	Extraneous microflora
	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>Bifidobacterium</i> sp.		
1	$8,0 \times 10^{12}$	$7,0 \times 10^{10}$	102	Not found
10	$7,7 \times 10^{12}$	$2,9 \times 10^{10}$	104	Not found
20	$6,0 \times 10^{12}$	$5,0 \times 10^9$	108	Not found
30	$1,0 \times 10^{11}$	$3,0 \times 10^9$	110	Not found
40	$1,2 \times 10^{11}$	$2,0 \times 10^9$	120	Not found
90	$1,7 \times 10^8$	$7,0 \times 10^8$	125	Not found

Table 8. Physicochemical and microbiological characterization of the probiotic milk during storage at $4 \pm 2^\circ\text{C}$

A technology for obtaining other probiotic foods - acidophilous milk and milk, containing *Lactobacillus acidophilus* and bifidobacteria - has been developed as well, which expands the range of dairy foods with preventive role for humans, which in turn is the key to protecting public health.

Lactic acid bacteria are applied in the production of different types of cheeses. Other microorganisms that form the specific properties of cheeses are involved as well. Using molds to obtain cheeses not only radically alters the organoleptic characteristics of cheeses, but also requires changes in the production technology. Depending on the types of microorganisms in the composition of starter cultures, cheeses with starter cultures of mesophilic lactic acid bacteria, starter cultures of mesophilic and thermophilic lactic acid bacteria and propionic acid bacteria, with the participation of molds, bifidobacteria and/or *Lactobacillus acidophilus* - dietetic (functional) cheeses [90] are obtained.

In the production of certain hard cheeses with high temperature of the secondary heating propionic acid bacteria participate in the formation of the specific taste, flavor and texture of the product along with lactic acid bacteria. Propionic acid bacteria absorb part of the lactate, forming propionic and acetic acid and carbon dioxide. Therefore, as a component of these starter cultures the propionic acid bacterial species *Propionibacterium frendenreichii* subsp. *frendenreichii*, *Propionibacterium frendenreichii* subsp. *shermanii* and *Propionibacterium frendenreichii globosum* are included.

The research on the development of starter cultures for yoghurt conducted by our research team shows the importance of achieving symbiosis between the strains in the composition of the starter culture for the quality of the finished product. Few strains of *L. bulgaricus* can be used to obtain a symbiotic culture. The symbiosis between *L. bulgaricus* and *Str. thermophilus* determines the taste-aroma complex of the finished products to a great extent.

A starter culture for hard cheese with the inclusion of the strain *Propionibacterium freudenreichii* subsp. *freudenreichii* NBIMCC 328 with high antioxidant activity (catalase, peroxidase and superoxide reductase), determined by the ORAC method (Oxygen Radical Absorbance Capacity), antimicrobial ability, moderate lipolytic and proteolytic activity is created. The ability of the microorganisms to neutralize free radicals is important for milk production and health, since they enter the gastrointestinal tract with food and their growth continues after intake. Thus another source of antioxidants (bacteria capable of synthesizing antioxidants during growth) is ensured.

Probiotic bacteria are included in a starter culture for hard cheese with high temperature of second heating (50-52°C), providing protection of the product in the process of maturation and storage.

At the end of the ripening process high content of beneficial microorganisms - lactic acid and propionic acid bacteria with concentration of 10^8 cfu/g - remains. There are no representatives of the pathogenic microflora. Extraneous microflora is less than 100 cfu/g. Moreover, in the final hard cheese the concentration of viable cells is more than 10^8 cfu/g. This opens up new paths for the usage of microorganisms with probiotic potential. The content of short-chain acids in the hard cheese with high temperature of secondary heating is determined by HPLC. The final product contains significant amount of propionate (14,9 mg/kg) and acetate (2 mg/kg).

Goat's milk improves blood composition and exhibits bactericidal properties, strengthens the immunity, accelerates the healing of bone traumas due to its significant levels of calcium, activates the work of the digestive glands and has anti-allergic properties. It also has a positive impact on diseases of the skin, joints, etc. It protects against tooth decay and helps build a healthy enamel. Gastric diseases are rapidly improved with goat's milk. In the cases of arthritis, rheumatism and all conditions in which acidic metabolic products occur predominantly such as diabetes, heart, lung, kidney, liver, etc. the health of the individual improves significantly after the inclusion of goat's milk in the diet. Goat's milk combined with soaked and peeled dates turns out to be useful combination in the case of gastric ulcer and in combination with dried figs in the case of arthritis.

Goat's milk is digested in the stomach 20 min after intake, unlike cow's milk, which requires 2 hours. Great part of the population eats goat's milk.

Yoghurts and yoghurt beverages from goat's milk with lactobacilli and bifidobacteria with probiotic properties are obtained as a result of the work of our research team (Table 9 and Table 10). They are characterized with high concentration of viable cells (above 10^8 cfu/cm³). Probiotic bacteria influence not only the functionality but also the flavor of these products.

Storage time Bio-yoghurt	10 days			20 days			30 days		
	N, [cfu/cm ³]		TA, [°T]	N, [cfu/cm ³]		TA, [°T]	N, [cfu/cm ³]		TA, [°T]
	LAB	bifidobacteria		LAB	bifidobacteria		LAB	bifidobacteria	
<i>Bif. bifidum</i> L1	5x10 ¹¹	3,2x10 ¹¹	97	4x10 ¹¹	8x10 ¹¹	110	6x10 ¹⁰	7x10 ⁸	108
MZ ₂ control	2,7x10 ¹²	-	68	3x10 ¹¹	-	112	3x10 ¹⁰	-	126
<i>Lactobacillus acidophilus</i> 2	1,3x10 ¹²	-	94	1,1x10 ¹²	-	105	6x10 ¹⁰	-	119
<i>Lactobacillus acidophilus</i> 2 + <i>Bif. bifidum</i>	9x10 ¹²	5,3x10 ¹¹	82	3,5x10 ¹²	4,2x10 ¹¹	112,5	6x10 ¹¹	4x10 ⁸	120

MZ₂ – starter culture for yoghurt containing a probiotic strain of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*

Table 9. Concentration of viable cells (N) and titrable acidity (TA) of goat yoghurt, produced with a probiotic starter cultures during storage

Storage time Beverage	1day			15 days			30 days		
	N, [cfu/cm ³]		TA, [°T]	N, [cfu/cm ³]		TA, [°T]	N, [cfu/cm ³]		TA, [°T]
	LAB	bifidobacteria		LAB	bifidobacteria		LAB	bifidobacteria	
<i>B. bifidum</i> L1	3,5x10 ¹²	1x10 ¹⁰	90	3,75x10 ¹¹	1,9x10 ¹⁰	98	4x10 ¹¹	1x10 ⁹	102
MZ ₂ control	7x10 ¹¹	-	62	6,58x10 ¹¹	-	90	5,8x10 ¹⁰	-	100
<i>Lactobacillus acidophilus</i> 2	5x10 ¹¹	-	58	5x10 ¹¹	-	96	3,8x10 ¹⁰	-	110

MZ₂ – starter culture for yoghurt containing a probiotic strain of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*

Table 10. Change in concentration of viable cells (N) of probiotic lactobacilli and bifidobacteria and titrable acidity (TA) of goat yoghurt beverages during storage at temperature 4±2°C

Probiotic goat yoghurt and yoghurt beverages have high concentrations of viable cells of lactobacilli and/or bifidobacteria and can be applied as probiotic foods for 30 days.

6.3. Probiotic bacteria in the composition of the starter cultures for fermented sausages without heating

Biological preservation of ground meat is an important and topical issue for the meat industry. Its solution is associated with the search for suitable strains of microorganisms that provide protective properties and pleasant taste and flavor of the finished products. By applying this method of preservation a number of advantages can be achieved, the most important of which are extending storage, usage of softer modes of cold storage, etc. To achieve targeted fermentation and quality maturation in the production of cured meat products starter cultures of lactic acid bacteria are imported. A new trend in the production of dried meat products is the inclusion of probiotic strains in the composition of starter cultures. They provide proper conduction of the fermentation process in meat foods and significant amounts of microflora beneficial to human health.

Meat products, which are not treated thermally are suitable carriers of probiotic bacteria [95, 96]. Strains of lactic acid bacteria with probiotic properties as starter cultures for fermented sausages are given in Table 11. These species are isolated from the gastrointestinal tract. Human digestive tract is a natural biological environment for *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* sp. These microorganisms are found in various fermented foods [90, 97, 98, 99]. According to Anderssen, 1998 [97], however, lactobacilli isolated from the intestines do not grow and contribute to the implementation of fermentation of the meat substrate.

In the preparation of starter cultures for the meat industry various microbial species are included (Table 9).

Microorganisms	Species
Bacteria	
Lactic acid bacteria	<i>Lactobacillus acidophilus</i> , <i>L.alimentarius</i> , <i>L.casei</i> , <i>L.curvatus</i> , <i>L.plantarum</i> , <i>L.pentosus</i> , <i>L.sakei</i> , <i>Lactococcus lactis</i> , <i>Pediococcus acidilactici</i> , <i>P.pentosaceus</i>
staphylococci	<i>Staphylococcus xylosus</i> , <i>S.carnosus</i> subsp. <i>carnosus</i> , <i>S.carnosus</i> subsp. <i>utilis</i> , <i>S.equorum</i> ; <i>Halomonadaceae</i> , <i>Halomonas elongata</i>
enterobacteria	<i>Aeromonas</i> sp.
<i>Bifidobacterium</i> sp.	
Actinomycetales	<i>Kocuria varians</i> <i>Streptomyces griseus</i>
Yeasts	<i>Debaryomyces hansenii</i> , <i>Candida famata</i>
Molds	<i>Penicillium nalgioevnse</i> , <i>Penicillium chrysogenum</i> , <i>Penicillium camemberti</i>

Table 11. Microbial species involved as components of starter cultures

A study conducted by Hammes et al., 1997 [100] clearly shows the beneficial effects of fermented meat products in the fermentation of which strains of the genus *Lactobacillus* with probiotic properties are used [100].

In studies conducted on representatives of the genus *Lactobacillus* it has been found that *Lactobacillus gasseri* JCM 1131T is suitable for meat fermentation. Moreover, *Lactobacillus gasseri* JCM 1131T and *Lactobacillus acidophilus* are the predominant species in the digestive tract of humans and *Lactobacillus gasseri* JCM 1131T has the ability to adhere to the gastrointestinal mucosa. Further research with this strain in meat environment shows some positive effects, but the culture is sensitive to the addition of NaCl and NaNO₂ and can only be used in meat products with low salt concentration without the addition of nitrites [101].

Lactobacillus sakei is widely used in meat industry as a species with probiotic properties, high antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*, and ability to retain the sensory profile of meat products [102, 103, 104].

The strains *Lactobacillus plantarum* NBIMCC 2415 [18] and *Pediococcus pentosaceus* NBIMCC 1441 are selected. They grow well in meat environment at high concentrations of sodium chloride and low temperatures, since under such conditions the processes of salting, ripening and drying of these products are performed. They also have well expressed fermentative activity without gas release, resistance to low pH, moderate proteolytic and lipolytic activity as well as antioxidant activity, which is associated with the formation of free amino acids, volatile fatty acids, carbonyl compounds and other substances that determine the taste and flavor of meat products, have good antimicrobial activity. The two strains are incorporated as starter cultures in a batch of sausage. *Lactobacillus plantarum* NBIMCC 2415 is imported as a monoculture with concentration of 10^8 cfu/g for the implementation of targeted lactic acid fermentation (batch I) and in a combination with *Pediococcus pentosaceus* NBIMCC 1441 (batch II). The microbiological parameters of the products are tested during the process of fermentation and drying. Experimental data are presented in Table 12 and Table 13.

Day	pH	Total Microbial Count, cfu/g	<i>S.aureus</i> , cfu/g	<i>Salmonella</i> sp., cfu/g	<i>E.coli</i> , cfu/g	<i>Enterococcus</i> sp., cfu/g	LAB, cfu/g
8	5,00	$1,1 \times 10^3$	-	-	3×10^3	$1,1 \times 10^3$	2×10^9
18	4,63	Under 10	-	-	-	$2,3 \times 10^3$	6×10^{11}
28	5,1	Under 10	-	-	-	Under 10	$7,8 \times 10^{10}$
40	5,5	Under 10	-	-	-	Under 10	8×10^8

Table 12. Microbiological parameters of the first batch of sausage in the process of fermentation and drying

Day	pH	Total Microbial Count, cfu/g	<i>S.aureus</i> , cfu/g	<i>Salmonella</i> sp., cfu/g	<i>E.coli</i> , cfu/g	<i>Enterococcus</i> sp., cfu/g	LAB, cfu/g
4	5,2	Under 10	-	-	-	Under 10^4	$8,4 \times 10^9$
14	5,5	Under 10	-	-	-	Under 10^2	$5,4 \times 10^{10}$
48	5,5	Under 10	-	-	-	Under 10	$3,5 \times 10^{10}$

Table 13. Microbiological parameters of the second batch of sausage in the process of fermentation and drying

The extraneous microflora is suppressed and the total number of microorganisms is reduced as well as the number of coliforms and enterococci, which ensures safety of the product on one hand and maintaining its quality during fermentation on the other. The product also contains a high concentration of viable cells (8×10^8 - $3,5 \times 10^{10}$ cfu/g) of the probiotic strain *Lactobacillus plantarum* NBIMCC 2415, which turns the product into a probiotic and healthy one, and these indicators increase its durability and storage time [18].

6.4. Probiotic bacteria in the composition of bread sourdough

Bread is one of the main products in the diet of contemporary people. The quality of bread depends upon several factors. Intrinsic parameters of the flour, such as carbohydrate [105, 106], gluten [107], mineral element [108], lipid content [109, 110] and endogenous enzyme activity [111], and on the other hand extrinsic parameters referring to the breadmaking procedure, such as temperature, stages and extent of fermentation [112], water activity [113, 114], redox potential and additives [115, 116, 117], and incorporation of nutritional or rheological improvers, such as dairy ingredients [118], affect the quality of the final product. The effect of these factors can be either direct or indirect, by affecting the microflora, either this is supplied as a commercial starter or in traditional sourdough processes. These factors influence the microflora submitted in the form of a starter culture or traditional processes involving sour dough [119].

Bread is considered to be perishable food, microbial spoilage is observed quite often. The growth of molds causing huge economic losses and reduction of the safety of the bread due to the production of mycotoxins. Fungal spoilage of wheat bread is mainly due to *Penicillium* sp., which cause around 90% of wheat bread spoilage [120]. Other common bread spoilage molds belong to the genera *Aspergillus*, *Monilia*, *Mucor*, *Endomyces*, *Cladosporium*, *Fusarium* or *Rhizopus* [121]. At present a number of alternatives are applied to prevent or minimize microbial spoilage of bread, e.g. modified atmosphere packaging, irradiation, pasteurization of packaged bread and/or addition of propionic acid and its salts [121, 122].

Propionic acid has previously been shown to inhibit moulds and *Bacillus* spores, but not yeasts to a large extent, and has therefore been the traditional chemical of choice for bread preservation [123]. Legislation implemented under the European Parliament and Council Directive No. 95/2/EC requires that propionic acid may only be added to bread in a concentration not exceeding 3000 ppm [124]. However, recent studies have shown that under these conditions propionic acid is not effective against common bread spoilage organisms [125]. Additionally, a reduction of preservatives to sub-inhibitory levels might stimulate the growth of spoilage molds [126] and/or mycotoxin production [127, 128, 129]. Recent trends in the bakery industry have included the desire for high-quality foods, which are minimally processed and do not contain chemical preservatives, thus increasing the interest toward natural preservation systems [130].

Among the natural means for preservation of bread is the use of strains of lactic acid bacteria, which are imported in the form of sourdough [131, 132], providing fast and reliable stability of the dominant microflora in the production cycle. As components of the starter cultures selected strains homo- and heterofermentative lactic acid bacteria are applied. The latter utilize substrates with the formation of lactic and acetic acid, resulting in acidification of the medium (pH, total titratable acidity (TTK)) [105, 131, 133]. Acetate production by heterofermentative metabolism is of major importance for the development of flavour. The molar ratio between lactic to acetic acid in bread (fermentation quotient, FQ) is considered optimum in the range between 2.0 and 2.7 [131]. Production of suitable end-products during

dough fermentation depends on the availability of soluble carbohydrates, which are attacked by the enzymes of the flour and the microbial enzyme systems [105, 134, 135, 136]. Metabolism of carbohydrates is species specific, even strain specific. It depends on the type of sugars, the co-presence of yeasts and the processing conditions [137].

Besides weak organic acids, i.e. lactic and acetic acid [138, 139, 140], LAB produce a wide range of low molecular weight substances [141], peptides [142] and proteins [143] with antifungal activity.

Sourdough is applied in the production of classic bread, sour bread, snacks, pizza and sweet baked goods. Sourdough fermentation increases the performance of the dough, improves the volume, texture, taste and nutritional value of the final product, slows down the loss of freshness and flavor and protects bread from mold and bacterial spoilage. These beneficial effects result from the appropriate balance between the metabolism of yeast strains and strains of hetero- and homofermentative lactic acid bacteria, which are the predominant microorganisms in natural sourdough. The metabolism of lactic acid bacteria is responsible for the production of organic acids and contributes, together with yeasts, to the production of aromatic components [144, 145, 146].

The activity of the lactobacilli in the composition of sourdough affects the protein fraction of flour during fermentation. This protein is particularly important for the quality of the bread, as the protein network of the bread determines its rheology, gas retention and thus the volume and texture of the bread. The substrates for the microbial conversion of amino acids in taste precursors and antifungal metabolites [147] are provided by proteolytic reactions. The levels of some peptides are reduced, which is helpful in the cases of inability to absorb cereal products by some people [148].

Bread with best quality is obtained by the simultaneous use of homo- and heterofermentative lactic acid bacteria in a certain ratio. Pure cultures of yeasts and lactic acid bacteria, imported in sufficient quantities provide fast and reliable stabilization of the dominant microbiota, normal fermentation process and actively participate in the quality of the finished bread. To observe this effect proper selection of species of lactic acid bacteria and process design, control over the purity and the activity of the cultures are required.

The strains *Lactobacillus casei* C, *Lactobacillus brevis* I, *Lactobacillus plantarum* NBIMCC 2415 and *Lactobacillus fermentum* J are isolated from naturally fermented sourdough, which defines their ability to grow in the mixture of flour and water, reaching high levels of viable cells and accumulating acid. Therefore, the growth of each of the four strains in the mixture of flour and water is examined. The change in the concentration of viable cells and the titratable acidity for 96 hours of cultivation at 30°C is traced. The proportions for the repeated kneading every 24 hours are: first day - 44% flour: 56% tap water and 5% 48-hour culture of the strain, second to fifth day: 25% starter from the previous day: 75% new mix flour / water with ratio 44% / 56%. All four strains of lactobacilli grow well in the mixture of flour and water, reaching 10^9 - 10^{15} cfu/g within 96 hours and the TTA of the sourdoughs increases to around 10^0 N (Table 14).

Time, h Strain	0 h		24 h		48 h		72 h		96 h	
	N, [cfu/g]	TTA, [°N]	N, [cfu/g]	TTA, [°N]	N, [cfu/g]	TTA, [°N]	N, [cfu/g]	TTA, [°N]	N, [cfu/g]	TTA, [°N]
<i>L.casei</i> C	2x10 ⁸	1,8	3x10 ¹¹	10,4	3,8x10 ¹¹	12	3,8x10 ¹²	11,2	8,1x10 ¹⁴	10,8
<i>L.brevis</i> I	7,6x10 ⁸	1,9	3x10 ¹⁰	13,2	4x10 ¹⁰	13	4,2x10 ¹⁰	8,6	5,6x10 ¹⁰	8
<i>Lactobacillus plantarum</i> NBIMCC 2415	2x10 ⁹	1,7	1,4x10 ¹¹	11,8	9x10 ¹²	11	1,8x10 ¹³	9	1,4x10 ¹⁵	8,8
<i>L.fermentum</i> J	1,3x10 ⁸	2	3x10 ⁹	9,6	5,2x10 ⁹	10,2	5,6x10 ⁹	8,8	7x10 ⁹	8

Table 14. Change in the concentration of viable cells (N) of lactobacilli and the total titrable acidity (TTA) of the medium in repeated kneading in flour/water mixture every 24 hours for 96 hours

Based on the results for the four strains of lactobacilli a starter culture for wheat bread is created by mixing them in a certain ratio. The ratio of is 2:1:1:1 = *Lactobacillus plantarum* NBIMCC 2415: *Lactobacillus casei* C: *Lactobacillus brevis* I: *Lactobacillus fermentum* J.

The accumulation of biomass and the change in TTA of the sourdoughs during the repeated kneading every 24 hours is determined. The following experiment scheme is applied: first day - 44% flour: 56% tap water and 10% of the combination; second to fifth day: 25% from the starter culture from the previous day : 75% new mix flour / water with ratio 44% / 56%. On the third day of repeated kneading yeasts are added to the sourdough (1g).

The results of the study on the starter culture for wheat bread are given in Table 15. The four strains develop with the accumulation of high concentrations of viable cells (over 10¹⁰cfu / g) of lactobacilli and TTA increases to 17,3°N.

In the sourdough molds have not been established. In addition to that, the metabolites formed by the lactic acid bacteria in the composition of the starter culture inhibit „wild” yeasts that get into sourdough through flours (Table 15). This ability is particularly important in sourdough fermentation of bread in repeated kneading for a long period of time - 6-9 months.

Time, h Starter culture	0 h				48 h				96 h			
	N [cfu/g]			TTA [°N]	N [cfu/g]			TTA [°N]	N [cfu/g]			TTA [°N]
	LAB	M	Y		LAB	M	Y		LAB	M	Y	
Wheat starter culture	3x10 ⁹	3x10 ¹	Under 10	2,5	6,2x10 ¹¹	nf	Under 10	8,4	2x10 ¹⁰	nf	Under 10	17,3

Table 15. Concentration of viable cells (N) of lactobacilli and of the Total Titrable Acidity (TTA) in the wheat starter culture and change in the microflora for 96 hours. LAB – lactic acid bacteria, M – molds, Y – yeasts, nf - not found

Along with determining the concentration of viable cells an organoleptic analysis of the starter culture is performed as well. The results show that for 48 to 72 hours of cultivation the starter culture reaches normal consistency of the sourdough and pleasant lactic acid flavor.

The starter culture is probated in industrial production - for the baking of bread 96-hour sourdough with different percentage is used; the percentage is determined by the weight of the used flour - 5%, 7% and 10%, according to the following scheme: 2 kg of flour, 1.5% NaCl, 2% yeasts, the respective percentage from the starter culture and tap water (the amount of water is determined by water absorption of the type of flour). Enhancers are added as well - 2 g/kg flour.

All the indicators of the sourdough and the bread are traced, so that the levels of incorporation of the sourdough would not adversely affect the rheological characteristics of the dough and the technologies adopted by manufacturers for the production of bread. The results of these experimental studies are presented in Table 16.

Wheat bread with the starter culture is baked as well as a control bread (without a starter culture). The data from the evaluation of the final bread with different percentages of the starter culture, including its strength and elasticity, the pieces of bread before and after baking, taste, flavor, etc. are shown in Table 16 and show acceleration of the fermentation process. The bread obtained with the starter culture is healthier, has more elasticity, the loaves of the bread are higher. The final wheat bread has softer and lighter crumb, with pleasant aroma and characteristic lactic acid odour.

Sample	1 Control (without starter culture)	2 Starter culture 5%	3 Starter culture 7%	4 Starter culture 10%
TTA of the starter culture	-	15,6	15,6	15,6
Dough		Elastic	Elastic	Elastic
Rise of the dough [min]	52	50	48	52
Amount of water [%]	53	51	50	48
Temperature of the dough [°C]	29.1	28.4	29.5	29.4
Pieces before baking		Higher than the control		
Rise of the dough [cm]	9.0	9.0	9.2	9.2
Baking (upper crust)	Normal	Normal	Normal	Normal
Aroma of the bread	Typical wheat bread aroma	Soft lactic acid aroma	Pleasant, characteristic lactic acid aroma	Strong and sharp characteristic lactic acid aroma
TTA of the bread	1.2	1.5	1.6	1.7

Table 16. Indicators characterizing the rheology of the sourdough, the flavor and aroma of the bread, prepared with 96-hour starter cultures

The created starter culture for sourdough for wheat bread improves its technological and organoleptic characteristics. Along with this it has been found to inhibit “wild” yeasts and mold spores in flour.

6.5. Soy probiotic foods

Soy foods are essential in the diet of the people in the Far East. They are rich in protein, supplying the body with all the essential amino acids for building and maintaining the tissues [149]. They are a source of flavones and isoflavones that exhibit antioxidant activity and can reduce the damage caused by free radicals [150]. Soybeans have stachyose and raffinose, oligosaccharides that are bifidogenic factors. The body is supplied with vitamins from groups B and D, mineral elements - calcium, magnesium, iron, etc. by traditional soy foods. Anti-cancer agents - protease inhibitors, saponins, phytosterols, phenolic acids, phytic acid and isoflavones, most of which are important flavones and isoflavones, which are polyphenolic compounds and relate to the group of plant estrogens, phytoestrogens, are also present in soy foods. The general term phytoestrogens refers to substances which have the effect of female hormones, but are not steroids. It is believed that soy foods play an important role in preventing chronic diseases such as menopausal disorders, cancer, osteoporosis, atherosclerosis.

Soy milk is obtained from dried, ripened, whole soybeans. They are soaked in fresh water for 16-18 hours at room temperature. The beans are washed, drained and ground. Hot potable water is added in a blender of Osterizer. The final suspension is filtered, autoclaved at 121°C, stored overnight at 5°C and it is processed to obtain soy milk products.

The dense residual mass is also rich in plant protein, vitamins C and E, calcium, manganese and iron and is a soy enrichment agent.

Soy milk contains no lactose. It replaces cow's milk for all people who suffer from allergies, lactase deficiency and milk protein intolerance. It can be used to carry out lactic acid fermentation with suitable strains of lactic acid bacteria (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Lactococcus lactis* ssp. *lactis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*) to obtain various fermented soy foods. It is a suitable environment for the development of new probiotic supplements. Having in mind the fact that it contains oligosaccharides, the obtained concentrates are synbiotics.

Soy milk yoghurt has been studied extensively [151, 152, 153]. Fermented soy milk products may provide economic and nutritional benefits, because they can be prepared at higher protein levels at comparable or lower cost than regular fermented milk products [154]. Soy proteins have favorable amino acid balance, meeting the essential amino acid, requirements, except for methionine [155]. The researches of a number of authors [156, 157, 158] show a lot of advantages of the soy milk products in the nutrition of children and adults, suffering from allergies, diabetes, cancerous, heart and renal diseases. Soy milk products

and soy milk yoghurt successfully replace fermented milk products from cow's milk [157, 158].

By selection of strains of lactobacilli (*Lactobacillus acidophilus* A) and bifidobacteria (*Bifidobacterium bifidum* L1) alone and in a combination with streptococci (*Streptococcus thermophilus* T3) soy probiotic milk and beverages, characterized by high concentration of active cells of lactobacilli and bifidobacteria (10^{11} - 10^{14} cfu/g) and moderate titratable acidity, which allows 20 days of storage under refrigerated conditions, are obtained.

It has been shown that the antioxidant activity of fermented soy foods is significantly higher in comparison with unfermented soy foods.

Wang et al., 2006 [149] explores the influence of spray-drying and freeze-drying on fermented soy milk with *L.acidophilus* and *Str.thermophilus* and bifidobacteria - *Bif. longum* and *Bif. infantis*. The authors demonstrate increased antioxidant activity in fermented soy milk and the increase is species specific. Freeze-drying of soy milk leads to lower reduction of the antioxidant activity. This opens up new opportunities to use soy milk for obtaining probiotic supplements and probiotic soy milks and beverages.

Soy cheese can be obtained from soy milk coagulated as a result of the action of lactic acid bacteria. Soy cheese is the result of fermentation with starter cultures for soy cheese and the probiotic strain *L.rhamnosus*.

Probiotic lactobacilli and bifidobacteria may be included in other non-fermented soy foods - soy mayonnaise, soy delicacies, etc.in concentration 10^6 - 10^7 cfu/g, which provides greater durability of soy foods.

Heenan et al., 2004 [159] includes *L.acidophilus*, *L.rhamnosus*, *L.paracasei* subsp.*paracasei*, *Sacch.boulardii* and *Bif.lactis* in concentrations 10^6 cfu/g in frozen non-fermented vegetable soy desserts made from soy beverage, sugar, butter, salt and stabilizers.

Thus, the durability of soy foods increases as well as their biological effect on the body since they deliver beneficial microflora as well. That is how the preparation of healthy foods without the application of chemical preservatives is achieved. The role of the chemical preservatives is conducted by the imported probiotic cultures.

6.6. Probiotic bacteria in the fermentation of fruit, vegetables, fruit and vegetable juices

Almost all fruits and vegetables can undergo natural fermentation as they are inhabited by many types of lactic acid bacteria. The latter vary as a function of the microflora of the raw material, the temperature and the storage conditions [160]. Currently fermented cabbage, olives, cucumbers, carrots, lettuce, peas, corn, tomatoes, onions, pickles, radishes, Brussels sprouts, etc. are being produced mainly by natural fermentation. They allow fermentation with starter cultures as well. Lactic acid bacteria including the probiotic strains that are included as components of the starter cultures for fermented

fruits and vegetables have the ability to grow in the fruit matrix and the cell vitality depends on the strain, the type of the substrate, the final acidity of the product [73], their resistance to high concentrations of salt in the medium, their ability to grow at temperatures around 18°C, to reproduce rapidly and to accumulate acids, which acidify the environment and inhibit the growth of extraneous microflora. Most of them belong to the genera *Leuconostoc* (*Leuconostoc mesenteroides*), *Lactobacillus* (*Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus casei*) and *Pediococcus* (*Pediococcus pentosaceus*) [161, 162] and can be used as monocultures and as combinations. During its growth in vegetable juice *Leuconostoc* helps the growth of other lactobacilli and bifidobacteria by synthesis of dextranase [163].

Different strains are characterized with different sensitivity to the pH of the juice, to the acidification as a result of the fermentation, to the metabolic products, to the environmental conditions such as temperature, etc. [164, 165]. It has been shown that the optimum temperature for the development of probiotic strains is 35-40°C and pH varies between 4,0 and 3,6 [6]. To protect the cells from the effects of the environmental factors agar, alginate, chitosan are used [165, 166, 167]. A probiotic banana product fermented with *Lactobacillus acidophilus*, included in alginate gel structures, is obtained. The inclusion of bacteria in alginate gel and carrageenan matrices protects the cells from the damages resulting from freezing and freeze-drying [168]. Encapsulation is applied in the production of probiotics as well [169].

Many fruits and vegetables allow processing to turn into media rich in nutrients, mineral elements, vitamins and antioxidants suitable for the growth of probiotic bacteria [170]. The probiotic strain *Lactobacillus plantarum* NBIMCC 2415 grows well in such medium (tomato juice) [18]. Tomato juice is a suitable medium for the growth of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* [170], which for 48 hours of growth at 30°C reach concentration of 10⁸cfu/ml. This probiotic beverage is kept at refrigerated temperature and maintains the amount of viable cells for 4 weeks. The same author obtained probiotic cabbage juice with the same strains of lactobacilli [171].

According to Rakin et al., 2007 [172] yeast autolysate can be added to vegetable juices before lactic acid fermentation. Its addition stimulates the growth of *Lactobacillus plantarum* and *Lactobacillus delbrueckii*.

Lactobacillus acidophilus and *Lactobacillus plantarum* can grow in red beet juice, reaching up to 10⁹ cfu/ml viable cells and reducing the pH from 6.3 to 4.5.

Of course during the growth of probiotic bacteria in fruit and vegetable juices it is possible to obtain a product with specific flavor and aroma. In such cases the addition of fruit juices, which remove the off flavor, is needed.

All this suggests that probiotic bacteria represent a potential for obtaining fruit and vegetable functional foods because of their ability to grow in them and their resistance to acidic environments.

7. Conclusion

Beneficial microorganisms (lactobacilli and bifidobacteria) interact with other members of the intestinal microflora. The ability of the selected strains of lactobacilli and bifidobacteria to inhibit the growth of most representatives of *Enterobacteriaceae* which cause toxemia and toxicoinfections and some molds is a criterion that the microbial strains in the composition of probiotics and probiotic foods must meet. This is particularly important for the industry because of the sustainability of their growth to the majority of antibiotics used in modern health care - while pathogenic microorganisms can develop polyvalent resistance towards antibiotics, they can not do so against probiotic bacteria. The antimicrobial effect of the beneficial microflora is due to the synthesis of lactic, acetic and other organic acids and bacteriocins (proteins associated with microbial cells).

The intact intestinal epithelium with normal intestinal microflora serves as a barrier to the migration of pathogens, antigens and other harmful substances from the intestinal contents. Thus the host is protected and normal functioning of the intestines is provided. The impaired balance of the gastrointestinal microflora leads to diarrhea, intestinal inflammation, problems with the permeability or activation of carcinogens from the intestinal contents.

The future will undoubtedly show the many benefits of the combination of compatible symbiotic bacterial strains and prebiotics in functional foods.

So far probiotics are an effective alternative to antibiotics and chemotherapy, but in the coming years they are expected to demonstrate their suitability as therapeutic and prophylactic agents for many diseases associated with disorders of the digestive system.

As far as the products themselves are concerned future studies should be directed towards the selection of strains of lactobacilli and bifidobacteria with high probiotic effect and the development of technologies for the production of improved probiotics and probiotic foods.

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