Living food™

RESEARCH ON QUALITY ASSESSMENT AND BIOFUNCTIONAL PROBIOTIC PRODUCTS OF THE COMPANY LIVING FOOD SP. Z O. O.

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PRODUCER'S PROBIOTIC ECOLOGICAL FOODSTUFFS

This study was created in cooperation with research workers of the University of Life Sciences in Poznań, Poland - dr hab. Daria Szymanowska from the Department of Biotechnology and Food Microbiology and dr hab. Joanna Kobus-Cisowska from the Department of Gastronomic Technology and Functional Food.

Uniwersytet Przyrodniczy w Poznaniu

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Living Food company was registered on March 9, 2010 and since the beginning of its activity specializes in the production of probiotic foods. The production was started with the assortment of milk-free concentrates and JOY DAY probiotic drinks, and thus far the company implemented a dozen of probiotic food preparations.

Living Food is the only company in the European Union producing probiotic food products in a liquid form, containing live, active probiotic bacteria, capable of immediately colonizing the digestive tract (mainly the gut). Excellent results are obtained thanks to the appropriate commitment of the staff and an innovative, cascade method of production specially selected sets of probiotic bacteria and their pro-health metabolites.

The entire production system and product control are based on the valid procedures in the implemented GMP, GHP and HACAP systems. Qualified and proven staff supervise the correctness of the system and quality of production.

1. LACTIC ACID BACTERIA - GENERAL CHARACTERISTICS

Lactic acid bacteria (LAB) are the group of microorganisms most commonly used in food production. They have many attractive features determining their use in industry (Gajewska and Błaszczyk, 2012). Moreover, some of them have confirmed pro-health properties (Słońska and Klimuszko, 2010). All LABs are gram-positive bacteria that do not produce catalase or spores (Libudzisz, 2013). LAB are characterized by high nutritional requirements (Jurkowski and Błaszczyk, 2012). They are capable of fermenting sugars under anaerobic conditions and as a result they synthesize lactic acid (Libudzisz, 2013). Due to the type of metabolism, LAB are divided into homo-and heterofermentative bacteria (Górecki and Bardowski, 2011), while due to the optimal temperature for their growth, into thermophiles and mesophiles (Jurkowski and Błaszczyk, 2012). Mesophiles produce lactic acid at a concentration of 1.5%, while thermophiles produce up to 3% (Libudzisz, 2013). The optimal pH for LAB is between 4 and 9.6 (Gajewska and Błaszczyk, 2012). All LAB are obligatory or optional anaerobic bacteria (Jurkowski and Błaszczyk, 2012). The microorganisms within this group show significant differences, which especially concerns those of the genus *Bifidobacterium.* They contain more G+C bases in their cells than other types of LAB (Libudzisz, 2013). The common habitat for LAB, depending on the species, are plants, milk, and some of them also inhabit the gastrointestinal tract of humans and animals (Górecki and Bardowski, 2011).

1.1 Systematics

LAB are classified into two different types, i.e., the thirteenth type-Firmicutes and the fourteenth type-*Actinobacteria*. Of all LAB, only those of the genus Bifidobacterium are included in the *Actinobacteria* type, while the others are of the thirteenth type. The genus *Bifidobacterium* belongs to the family *Bifidobacteriaceae*, of the order Bifidobacteriales and of the class Actinobacteria. The Firmicutes type includes two orders in which the rest of the LAB is classified. The first order is *Bacillus*, which includes only one family - *Sporolactobacillaceae* and one type of lactic acid bacteria - *Sporolactobacillus.* The second order is *Lactobacillales,* which consists of six families and thirty-three types of lactic bacteria. The first family is *Lactobacillaceae* and its genera: *Lactobacillus, Paralactobacillus* and *Pediococcus.* The second is *Aerococcaceae,* which includes seven types of bacteria: *Aerococcus, Abiotrophia, Dolosicoccus, Eremococcus, Facklamia, Globicatella* and

Ignavigranum. Twelve genera of LAB are included in the third order *Carnobacteriaceae*: *Carnobaterium, Alkalibacterium, Allofustis, Alloiococcus, Atopobacter, Atopostipes, Desemzia, Dolosigranum, Granulicatella, Isobaculum, Marinilacobacillus* and *Trichococcus.* The fourth family is called *Enterococcaeae* and includes the genera *Enterococcus, Melissococcus, Tetragenococcus* and *Vagococcus.* Leuconostocaceae, i.e., the fifth family includes only three genera: *Leuconostoc, Oecococcus* and *Weisella*. The last one is *Streptococccaceae* and its genera: *Streptococcus, Lactococcus* as well as *Lactovum*. All lactic bacteria of the Firmcutes type are included in one class *Bacilli* (Moneta and Piątkiewicz, 2010; Gajewska and Błaszczyk, 2012; Jurkowski and Błaszczyk, 2012; Vos et al., 2009).

1.2 Metabolism

Due to nutrients utilization, LAB can be classified as homo-and heterofermentative ones. Obligatory homofermentative bacteria produce mainly lactic acid, but also low concentrations of other metabolites. In the case of heterofermentative bacteria, in addition to lactic acid, a number of other substances such as acetic acid, ethanol, carbon dioxide, diacetyl are also synthesized (Trojanovska et al., 2009). A form of lactic acid synthesized by lactic acid bacteria--L(+) or D(-)-is also strain-dependent (Narayanan et al., 2004). The main raw material used by LAB are carbohydrates, mainly six carbon sugars (Gajewska and Błaszczyk, 2012). Both types of bacteria also have the ability to ferment lactose (Libudzisz, 2013). Homofermentative LAB metabolize glucose in the Embden-Meyerhof-Parnas pathway (EMP) (Jurkowski and Błaszczyk, 2012). Two pyruvate molecules are formed in the final stage of glycolysis, which, as a result of the redox reactions, are reduced to the final fermentation product, i.e., lactic acid (Gajewska and Błaszczyk, 2012). In the case of heterofermentation, glucose is fermented in the pentosophosphate pathway. This difference is due to the fact that heterofermentative LAB do not have certain enzymes necessary for the glycolysis process. As a result of the occurring reactions, glucose-6-phosphate, 6-phospho-gluconic acid, then carbon dioxide and ribulose-5-phosphate are produced. The latter is converted into xylose-5-phosphate from which 3-phosphogliceric aldehyde and acetylphosphate are obtained. The first compound produces lactic acid and the second, depending on environmental conditions, acetic acid or ethanol. The presence of oxygen is necessary in order to form acetic acid, while ethanol is formed in the absence of oxygen (Libudzisz, 2013; Jurkowski and Błaszczyk, 2012). It is worth mentioning that LAB, apart from metabolizing sugars, also have the ability to ferment other compounds such as fats, which consequently affects the aroma of dairy products. On the other hand, the shortage of amino acids necessary for growth mobilizes LAB to degrade proteins present in the environment. This mechanism also has a positive impact in the context of food industry, as it enables the acquisition of appropriate organoleptic characteristics (Gajewska and Błaszczyk, 2012).

1.3 Characteristics of LAB genera

Genus *Lactobacillus*

Bacteria of the genus *Lactobacillus* are LAB belonging to the *Bacilli* class, of the order *Lactobacillales* and family *Lactobacillaceae* (Moneta and Piątkiewicz, 2010). They are in the form of sticks and are included in the optional anaerobes (Jurkowski and Błaszczyk, 2012). They do not produce endospores and catalase (Słońska and Klimuszko, 2010), they are acidophilic (Trojanowska et al., 2009). Bacteria of this type are structurally diverse (Libudzisz, 2013). Bacteria of the genus *Lactobacillus* are divided into three types due to the synthesized metabolites. The first one includes homofermentative bacteria. The second type are relatively heterofermentative bacteria, while the last group includes absolutely heterofermentative microorganisms (Słońska and Klimuszko, 2010). Both thermophilic and mesophilic strains occur in this type (Libudzisz, 2013). They occur naturally in decomposing plant raw materials or fecal matter, inter alia, due to the fact that they are an element of the intestinal microbiome of humans and animals (Gajewska and Błaszczyk, 2012). Due to their properties, they are widely used in the food industry. Bacteria of the genus *Lactobacillus* are used for the production of fermented milk drinks, sauerkraut and pickled cucumbers or bread (Libudzisz, 2013; Trojanowska et al., 2009). The genus *Lactobacillus* has the status of GRAS (generally recognized as safe) (Słońska and Klimuszko, 2010), which means “safe to use.” Some strains belonging to this type have proven pro-health properties and are classified as probiotics. These are species of *Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus johnsonii* or *Lactobacillus plantarum* (Gajewska and Błaszczyk, 2012). It is also worth mentioning that the bacteria of the genus *Lactobacillus* are used on an industrial scale for bacteriocins production (Steinka, 2009).

Our products also include representatives of the genus *Lactobacillus.*

- ***Lactobacillus rhamnosus* GG (ATCC 53103)** – is characterized by a positive effect on the immune system (anti-allergic properties) and digestive system, including increased effectiveness of inflammatory bowel disease treatment, decreased incidence of diarrhea. Supplementation of products containing *L. rhamnosus* GG strain has a positive effect on the outcomes of therapy against *Helicobacter pylori* and its administration to infants favors their good development.
- ***Lactobacillus rhamnosus* LR 04 (DSM 16605)** – is highly effective in the treatment of chronic diarrhea in the elderly, contributes to a reduction in the incidence and severity of respiratory diseases, has a strong antimicrobial activity against *E. coli* strains.
- ***Lactobacillus rhamnosus* LR 05 (DSM 19739)** - is characterized by anti-inflammatory and immunological properties, used in the treatment of allergies.
- ***Lactobacillus casei* 101/37 (LMG P- 17504)** – is characterized by immunomodulatory properties and contributes to IBS symptoms alleviation.
- ***Lactobacillus acidophilus* LA 1 (LMG P- 21904)** - contributes to maintaining a healthy digestive tract in patients with ulcerative colitis.
- ***Lactobacillus delbrueckii* ssp. *bulgaricus* LB 2 (LMG P-21905)** – is used to maintain remission in patients with ulcerative colitis, contributes to the alleviation of IBS symptoms.
- ***Lactobacillus plantarum* LP 02 (LMG P- 21020)** – cis characterized by antimicrobial activity against E. coli strains; contributes to a reduction in the incidence of respiratory diseases.
- ***Lactobacillus plantarum* LP 01 (LMG P-21021)** – is characterized by properties associated with alleviation of hypersensitive bowel syndrome symptoms and allows to increase the frequency of bowel emptying in children with functional constipation; it is characterized by strong antimicrobiological properties against strains of *E. coli* bacteria.
- ***Lactobacillus fermentum* LF 2 (LMG 27299)** – chas the ability to produce high concentrations of exopolysaccharide (EPS), which consequently contributes to lowering blood cholesterol levels, anticancer, anti-ulcer, and immunomodulatory properties.

Genus *Bifidobacterium*

Bifidobacterium genus differs significantly in phylogenetic terms from another LAB (Jurkowski and Błaszczyk, 2012). These bacteria belong to the Actinobacteria class, of the order *Bifidobacteriales* and family *Bifidobacteriaceae* (Moneta and Piątkiewicz, 2010). These are thermophilic sticks (Samet and Bronk, 2008; Libudzisz, 2013), included to absolute anaerobic bacteria (Jurkowski and Błaszczyk, 2012). *Bifidobacterium* undergoes lactic heterofermentation, which results in the production of acetic acid and lactic acid (Zielińska et al., 2012). *Bifidobacterium* bacteria naturally colonize human and animal intestines from where they are isolated (Libudzisz, 2013). They are characterized by proven beneficial effects on the human body and are classified as probiotics (Steinka, 2011). In the food industry, they are mainly used in the dairy industry (Libudzisz, 2013).

Our products also include representatives of the genus Bifidobacterium.

- ***Bifidobacterium breve* BL 10 (LMG P- 17500)** – positively evaluated in remission maintaining in patients with ulcerative colitis, contributes to the alleviation of IBS symptoms
- ***Bifidobacterium breve* Bbr 8 (LMG P-17501)** – is characterized by positive effect in remission maintaining in patients with ulcerative colitis; contributes to the alleviation of IBS symptoms
- ***Bifidobacterium animalis* subsp. *lactis* Bi 1 (LMG P- 17502)** – is characterized by positive effect in remission maintaining in patients with ulcerative colitis; contributes to the alleviation of IBS symptoms
- ***Bifidobacterium longum* BL 03 (DSM 16603)** – contributes to an increased frequency of bowel movements in children with functional constipation

Genus *Streptococcus*

The bacteria of the species *Streptococcus salivarius* ssp. thermophilus, formerly *Streptococcus thermophilus,* belong to the type *Firmicutes*, class *Bacilli,* order *Lactobacillales,* family *Streptococaceae,* genus *Streptococcus.* These are gram-positive, thermophilic bacteria with an optimal growth temperature of 45°C, included into streptococci. *Streptococcus salivarius* ssp. Thermophilus bacteria are used in the food industry, in the production of yoghurts, cheese and other fermented products. The main effect of this group of bacteria metabolism is the biosynthesis of lactate from lactose, thus, they function very well in the

milk environment. *Streptococcus salivarius* bacteria produce bacteriocynogenic factor and urease and are able to synthesize polysaccharides (e.g. hyaluronic acid) and tolerate oxygen environment.

Our products also include representatives of the genus *Streptococcus.*

- ***Streptococcus thermophilus* 9Y (LMG P-17225)** – is active in modulating the immune response, contributes to remission maintaining in patients with ulcerative colitis and alleviates IBS symptoms
- ***Streptococcus thermophilus* Z 57 (LMG P-21908)** - contributes to the alleviation of IBS symptoms
- ***Streptococcus thermophilus* FP 4 (DSM 18616)** - promotes the regeneration of the body after training and contributes to the reduction of muscle tension after strength exercise

Genus *Leuconostoc*

The genus *Leuconostoc* are grains which, like *Lactobacillus,* are included in the *Bacilli* class and of the order of *Lactobacillales.* However, they differ in family, as *Leuconostoc* belongs to the *Leuconostocaceae* family (Moneta and Piątkiewicz, 2010; Jurkowski and Błaszczyk, 2012; Trojanowska et al., 2009). These are gram-positive obligatory heterofermentative mesophiles (Trojanowska et al., 2009; Libudzisz, 2013). They are relative anaerobic and catalase-negative bacteria (Hemme and Foucaud-Scheunemann, 2004). The main metabolites produced by bacteria of the genus *Leuconostoc* are lactic acid as enantiomer D(-) and ethanol (Libudzisz, 2013). Commonly, bacteria of the genus *Leuconostoc* occur on the surface of green plants (Hemme and Foucaud-Scheunemann, 2004). They are used in the dairy industry for the production of fermented milk drinks, cream, maturing cheese or butter (Holland, 2011). Some strains have the ability to produce carbon dioxide, which causes holes formation in the cheese (Libudzisz, 2013). *Leuconostoc* bacteria synthesize diacetyl, which is responsible for the characteristic "buttery" aroma (Holland, 2011). Sometimes the presence of bacteria of the genus *Leuconostoc* is unfavorable. They may be a mucosal factor in sugar industry products (Hemme and Foucaud-Scheunemann, 2004).

Genus *Lactococcus*

The genus *Lactococcus* represents a gram-positive bacteria, as well as other LAB in the *Bacilli* class and the order *Lactobacillales.* In this case the bacteria belong to the *Streptococcaceae* family (Moneta and Piątkiewicz, 2010) (Trojanowska et al., 2009). They perform milk homofermentation and are classified as mesophiles (Jurkowski and Błaszczyk, 2012) (Petrov et al., 2008). The main metabolite produced by this type of bacteria is L(+) lactic acid (Petrov et al., 2008). *Lactococcus lactis* is a species of the genus *Lactococcus* that is important in food processing (Petrov et al., 2008). It is mainly used by the dairy industry, but also participates in the production of fermented plant raw materials (Odamki et al., 2011; Libudzisz, 2013). Moreover, the *Lactococcus lactis* species is a producer of nisin, used in the food industry as a food preservative (Jurkowski and Błaszczyk, 2012; Journal of Laws No. 232 item 1525). Additionally, its positive influence on the survival of *Bifidobacterium* bacteria was found (Yonezawa et al., 2010).

1.4 The use of LAB in food industry

LAB have been used in food processing for hundreds of thousands of years. Originally, people did not realize that microorganisms are responsible for the formation of various types of products (Czarnecki and Czarnecka, 2006). Today, microorganisms are widely used in all branches of the food industry. They allow to obtain an extensive assortment of food products with specific taste and smell characteristics and positive effect on human body functioning (Babuchowski and Wzorek, 2003). LAB can be found in fermented milk drinks as well as in cold meats (Chabłowska et al. 2009). Most often, LAB cultures are added to the raw material subjected to processing in the form of primers, containing carefully selected strains with a specific effect (Jurkowski and Błaszczyk, 2012). Sometimes spontaneous fermentation is carried out with the participation of microorganisms naturally present in the raw material, however, in this case there is no full control over the course of the process, which in turn may make it impossible to obtain a series of products with identical properties (Zaręba and Ziarno, 2011). However, autochthonous microflora of the raw material is often introduced into starter cultures (Chabłowska et al. 2009). LAB produce many metabolites, which have a preservative effect on food. An example is lactic acid produced by them, which significantly lowers the pH of the environment, which in turn prevents the development of undesirable microflora. Hydrogen peroxide or bacteriocins,

in particular nisin, are also important. Moreover, LAB prevent vitamin degradation (Zaręba and Ziarno, 2011) and increase nutrients absorption. Particularly important are LAB of probiotic character, allowing to obtain food products with pro-health properties (Libudzisz, 2013).

1.4.1 Dairy industry

The dairy industry to the highest degree uses the potential of LAB. Such products are also very popular among consumers, especially as they are characterized by great diversity (Nowak et al., 2007). They occur in both solid and liquid form. The most popular products of the dairy industry that can be found in solid form include various types of hard cheese and cottage cheese. On the other hand, liquid products include kefir, buttermilk, kumys, yogurt, acidified milk (Babuchowski and Wzorek, 2003) and beverages such as bio-yogurt, acidophilic milk or bifidus milk (Grzegorczyk, 2010). Milk fermented drinks are divided into four generations. The first generation includes, inter alia, curdled milk obtained by spontaneous fermentation of milk autochthonic microorganisms, i.e., bacteria belonging mainly to the genus *Lactobacillus* (Libudzisz, 2013). The second type are products such as kefir, yogurt or kumys. They are produced with the use of appropriate starter cultures (Mojka, 2013). Both kefir and kumys are produced with the use of lactic-alcohol fermentation process. They differ mainly in the raw material from which they are made. Kumys are traditionally made from mare's milk or camel's milk, while kefir is made from cow's milk (Kołożyn-Krajewska and Sikora, 2004; Mojka, 2013). The bacteria used in the production of kefir are most often those of the genera *Lactobacillus* and *Leuconostoc,* while for kumys the most popular is *Lactobacillus* delbrueckii sp. bulgaricus (Stankiewicz, 2009). However, in order to obtain a popular yogurt, the species *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are added (Babuchowski and Wzorek, 2003). The third generation drinks, in addition to the species already mentioned above contain the addition of probiotic bacteria such as *Lactobacillus casei* (Mojka, 2013). The consumer can easily recognize third generation beverages on the market, since they have the prefix "bio" (Kołożyn-Krajewska and Sikora, 2004). The fourth generation of fermented lactic drinks includes acidophilic milk and bifidus milk. They are produced exclusively with the use of probiotic LAB (Jałosińska, 2007). Acidophilic milk is obtained thanks to the process carried out by Lactobacillus acidophilus (Stankiewicz, 2007), while bifidus milk with the participation of bacteria of the genus *Bifidobacterium* (Grzegorczyk, 2010).

Fermented products of the dairy industry of solid consistency are obtained due to addition of carefully selected starter cultures. They are mainly used in the production of hard matured cheese and cottage cheese. Hard cheeses contain *Lactococcus* bacteria (Libudzisz, 2013). Depending on the type of cheese, other LAB such as *Lactobacillus* sticks, molds and yeasts are also used (Babuchowski and Wzorek, 2003). In particular, *Lactocococcus* lactis, which are responsible for the coagulation process, are used in the production of cottage cheese (Babuchowski and Wzorek, 2003).

1.4.2 Fermented plant raw materials

Both fruit and vegetables are an essential part of every person's diet. They provide many nutrients necessary for the proper functioning of the human body (Babuchowski and Wzorek, 2003). It is recommended to consume them in the amount of five servings a day, constituting a total of about 400 grams (http://www.kups.org.pl). Unfortunately, these are raw materials that remain fresh for a short time and deteriorate very quickly (Lada, 2008). Therefore, there is a search for ways to extend their shelf-life. One of such methods is lactic fermentation of plant raw materials (Babuchowski and Wzorek, 2003). This form of preservation is widely accepted by the consumers due to the confirmed safety of consumption associated with its long heritage of use (Trząskowska, 2013). Apart from preservative activity, fermentation carried out by LAB also allows to obtain new groups of products not only with attractive sensory characteristics, but also with high content of vitamins, antioxidants and other important compounds, which in the case of other types of preservation, e.g. heat treatment, may be subject to degradation (Zaręba and Ziarno, 2011). Most often, pickled products are obtained by lactic fermentation of plant raw materials. The most popular in our country are pickled cucumbers and cabbage, also pickled beets can be found quite often (Babuchowski and Wzorek, 2003). Fruit and vegetable juices are also fermented in Poland and many other countries. These products are still gaining popularity due to the problem of lactose intolerance found in part of the population and the growing number of people following a vegan diet (Zaręba and Ziarno, 2011). Increasingly, preservation by acidification is used in the production of animal feed on organic farms. In this way the feed is protected against the development of undesirable microflora and its consumption has a positive effect on the nutritional status of the animals (Zielińska et al., 2013). The native microflora of the raw material is generally used in the produc-

tion of silages (Libudzisz, 2013). Usually species such as *Lactobacillus brevis* or *Lactobacillus plantarum* as well as *Leuconostoc mesenteroides* are involved in these processes (Trojanowska et al., 2009). *Lactococcus lactis* and *Lactobacillus pentoaceticus* species are often present in cabbage (Babuchowski and Wzorek, 2003). In the case of juices, an appropriate starter culture is prepared. Its composition depends on the type of processed fruit and vegetables, but most often these are strains classified as probiotics (Zaręba and Ziarno, 2011). As mentioned earlier, LAB have a positive effect on the quality of the final products obtained. The main preservative effect is shown by lactic acid, which is a product of LAB metabolism (Chabłowska et al. 2009). Its activity is directly related to an increase in environment acidity (Lada, 2008). It is formed as a result of carbohydrates metabolism. In addition, it gives silage a crisp taste, stops the development of pathogenic microflora in the intestines, concurrently stimulating the growth of normal intestinal bacteria (Chabłowska et al., 2009). In addition, fermented plant raw materials may have an increased content of vitamins compared to the raw materials from which they were obtained (Szydłowska and Kołożyn-Krajewska, 2010). They also obtain a characteristic smell (Libudzisz, 2013) and, what is important in the case of diet, they are products with reduced calorific value (Szydłowska and Kołożyn-Krajewska, 2010).

1.4.3 Bread fermentation

Bread is most often made from wheat flour and rye flour. In Poland, bread made from mixed flour, i.e., wheat-rye flour, enjoys the greatest recognition (Chabłowska et al. 2009). In the case of wheat bread production, the main role is played by yeast and alcoholic fermentation carried out by yeast. On the other hand, LAB contribute to the formation of rye bread and products obtained with the use of non-bread flour, such as oats or barley. These differences are due to the specific characteristics of both flours (Babuchowski and Wzorek, 2003; Reps, 2003). LAB, and more precisely the metabolites produced by them, determine the specific taste and smell characteristics, as well as improve the nutritional value and better digestibility of mineral components (Piasecka-Jóźwiak et al., 2006). Specially selected starter cultures are used for the production of rye or mixed bread (Kawka et al., 2007). Only autochthonous flora of flour is rarely used (Piasecka-Jóźwiak et al., 2006). The starter cultures used in the production of rye and mixed bread mainly consist of *Lactobacillus* bacteria and yeasts, where their numerical ratio is 100:1 (Reps, 2003). Only their synergistic effect allows to obtain high quality bread. The species *Lactobacillus sanfranciscensis* and *Lactobacillus plantarum* are of particular importance. The first one hydrolyzes maltose, while the second one affects the elasticity and strength of the flesh (Libudzisz, 2013). In addition, starter cultures consist mainly of *Lactobacillus, Lactococcus* and *Leuconostoc* bacteria (Piasecka-Jóźwiak et al., 2006). The task of lactic bacteria is to acidify the dough, which, in addition to giving it a characteristic taste, also guarantees the desired structure of the product obtained (Libudzisz, 2013). Bread containing non-bread cereals is still gaining in popularity. Both oats and barley have a very beneficial effect on human health, they can improve health status with already existing abnormalities (high cholesterol or weight disorders) (Rozmierska et al., 2013; Kawka et al., 2007).

1.5 Main metabolites of LAB

Lactic acid

Lactic acid is a metabolite produced by LAB (Strus, 1998). It is used in many processing industries, including food processing (Jurkowski and Błaszczyk. 2012). Its characteristic activity involves an acidification of the environment. Reduced pH inhibits the development of undesirable microflora in food products (Śliżewska et al., 2006). Its strong preservative activity is effectively used in meat or fruit and vegetable processing (Walczycka, 2005; Trojanowska et al., 2009). It is an additional substance, legally permitted for use in food and defined by the symbol E 270 (Journal of Laws No. 232 item 1525). L(+) lactic acid enantiomer is the most desirable in industrial production (Libudzisz, 2013). It is produced mainly by homofermentative LAB belonging to the genus *Lactobacillus* (Babuchowski and Wzorek, 2003) and in particular the species *Lactobacillus casei* and *Lactobacillus delbruckii* (Trojanowska et al., 2009). Glucose, sucrose and waste from the sugar or cheese industry are generally used as raw materials for lactic acid production (Libudzisz, 2013).

Dextrans

Dextrans are produced by lactic fermentation bacteria. They belong to EPS, i.e., bacterial exopolysaccharides. They are classified as α-D-glucans, which are homopolysaccharides (Górska et al., 2007). They are produced by LAB belonging to the *Streptococcus* and *Leuconostoc genera,* mainly *Leuconostoc mesenteroides* (Quader et al., 2005). Unfortunately, dextran produced by LAB of *Leuconostoc* genus

negatively affects the properties of diffusion juices, hence the presence of these bacteria in the sugar industry is avoided (Libudzisz, 2013). In the food industry, this compound is sometimes used to fix syrups (De Vuyst and Degeest, 1999).

Bacteriocins

Bacteriocins are another metabolic products of gram-positive LAB (Steinka, 2009). They are divided into four classes. The most popular of them is nisin that belongs to the first class, i.e., the lantibiotics (Jurkowski and Błaszczyk, 2012). This substance is the only one among all bacteriocins to have GRAS status, which confirms the safety of its use. In the Ordinance of the Minister of Health of 22 November 2010 on permitted additional substances, as amended on 22 April 2011, nisin was defined as a preservative with symbol E 234. It also includes information about its maximum permitted dose in individual food products (Journal of Laws No. 232 item 1525; Journal of Laws No. 91 item 525). In general, these compounds do not pose a threat to human health as they are hydrolyzed by digestive tract enzymes to easily assimilable and harmless amino acids (Gwiazdowska and Trojanowska, 2005). Bacteriocins have strong antimicrobial properties, so they are used in many branches of the food industry, for example in the fermentation and dairy industry (Jurkowski and Błaszczyk, 2012). In addition, they also have properties preventing the development of filamentous fungi (Gwiazdowska and Trojanowska, 2005; Steinka, 2009). It is worth noting that apart from nisin, bacteriocins such as reuterine, lactocin or saccine are also used in meat processing. The basic direction of their use is to inhibit the development of *Listeria monocytogenes* (Walczycka, 2005). The main producers of bacteriocins on an industrial scale are bacteria of the genera *Lactobacillus, Lactococcus* and *Leuconostoc.* Only bacteriocins produced by LAB are treated as harmless by the food industry (Steinka, 2009). *Lactococcus lactis* are responsible for the production of nisin (Gwiazdowska and Trojanowska, 2005). Nisin has an inhibitory effect on various types of bacteria, both LAB and typical food pathogens such as *Salmonella, Listeria* or *Clostridium.* In the case of the latter, it additionally prevents production of spores by them (Słońska and Klimuszko, 2010).

1.6 Probiotic preparations

According to the current FAO/WHO definition, probiotics are living microorganisms that have a positive effect on the consumer's organism (Nowak et al., 2010). Probiotics include LAB naturally present in the human gastrointestinal tract from which they are isolated (Libudzisz, 2004). The most important pro-health activity of probiotics is based on the settlement of the gastrointestinal tract, increasing the immunity of the organism, minimizing the negative effects of antibiotics, such as the occurrence of diarrhea, prevention of hypercholesterolemia or production of enzyme decomposing lactose, which is particularly beneficial for people suffering from intolerance of this disaccharide. They are also attributed with anticancer and many other useful properties (Steinka, 2011; Heczko et al., 2005; Jach et al., 2013). These preparations also have an effect on animal organisms, so they are often used in their feeding (Reps, 2003). In order for a strain to be considered probiotic, it is necessary to prove its pro-health properties scientifically (Libudzisz, 2013). Currently, they are confirmed for a small number of bacterial species, mainly belonging to the genera *Lactobacillus* and *Bifidobacterium. Bifidobacterium animals, Bifidobacterium lactis* Bb-12, *Lactobacillus johnsonii* LA1, *Lactobacillus acidophilus* CRL639, *Lactobacillus casei* Shirota, *Streptococcus thermophilus* and others are the most common strains in food industry (Jach et al., 2013; Heczko et al., 2005). Probiotic preparations are usually referred to as additives and are considered as food (Jach et al., 2013). They are most often added to fermented milk drinks (Libudzisz, 2013), but their range of use is constantly increasing. They are often found in products of plant origin, e.g. various kinds of juices (Zaręba and Ziarno, 2011). Meat industry is also conducting research on their use in the production of cold meats (Staruch and Walczycka, 2011). They are usually added to food in the form of freeze-dried products (Jack et al., 2013). Their quantity and the method of technological processing is important for the proper functioning of LAB of probiotic character. It is assumed that the minimum number of living probiotic bacteria in the finished product is 106 cfu/g or mL of the product (Trafalska and Grzybowska, 2004).

1.7 Literature

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RESULTS OF RESEARCH ON THE QUALITY AND PRO--HEALTH POTENTIAL OF PROBIOTIC PRODUCTS

1. Product quality assessment

One of the key factors that determines the commercial success of a product is its quality. In the case of liquid probiotic products, the quality is determined by the number of microorganisms throughout the shelf-life, their vitality and the sensory characteristics of the product preserved.

1.1 Microbial count in our products

Appropriate amount of probiotic microorganisms in the product determines its biofunctionality and also determines its market position. It is worth noting that Living Food sp. z o.o. has an actively working microbiological laboratory since 2018. Modern equipment and professional staff watch not only over the quality of products but also over the hygienic condition of the whole plant every day. Microbiological analyses of our products are performed both for de-

sired microorganisms such as *Lactobacillus* bacteria, *Bifidobacterium* bacteria, but also for undesirable microorganisms such as yeasts, molds, *Salmonella* sp. bacteria, *Listeria* sp. bacteria or coliforms. The products are analyzed already at the production stage (product multiplication and stabilization) and also after the bioprocess as well as, selectively, at the stage of storage.

Methods

The number of microorganisms is determined using the Koch flooding method. One gram of the sample is suspended in 90 mL of 0.9% saline solution and shaken vigorously. Decimal dilutions of 10^{-1} to 10^{-8} are made from the resulting cell suspension. 1 mL of the solution is taken from the selected dilutions and applied to the sterile Petri dishes. The dishes are then flooded with liquefied and adequately cooled media: MRS agar, BSM agar, PD agar, glucose enriched agar. After solidification, the samples are incubated for 48–72 h at a temperature appropriate for a given group of microorganisms. LAB and yeasts were cultured under aerobic conditions, while Bifidobacterium bacteria were cultured under anaerobic conditions using anaerostats.

Conclusions

- The qualitative and quantitative composition of our products meets the requirements for probiotic products.

1.2 Macroscopic evaluation of probiotic bacteria colonies

The phenotype of bacterial colonies indicates their physiological state, including vitality. Moreover, each group of microorganisms is characterized by a specific type of growth both in liquid and solid media. Many years of experience and insightful observation of bacterial colony phenotype often allows to characterize the physiological state of the microorganisms that form it. Well visible colonies with intense color and typical smell prove good condition of microorganisms. Small, poorly visible colonies may indicate that the microorganisms included in them have been exposed to environmental stress either at the culture stage or during the assay. The physiological state of probiotic microorganisms cells is a insignificant issue. Even if the number of cells in the product is sufficient, poor vitality may consequently make it impossible to perform the desired function in the body.

Since we have the company's microbiological laboratory, we observe bacterial colonies and cells in a microscopic image, which allows us to assess the condition of microorganisms both during the production process and during the storage of finished products.

Methods

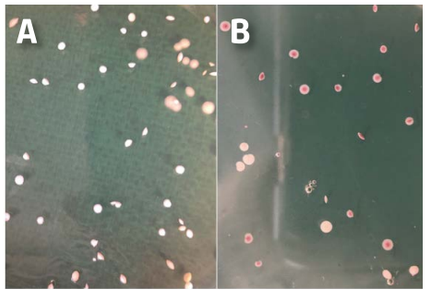
The method of reduction culture on MRS agar or BSM agar media is used for macroscopic evaluation of microorganisms. Particular attention is paid to single colonies in the third and fourth zones of the culture. The colony description takes into account indicators such as size, diameter, shape, edge, area, transparency, color, colony surrounding and growth character.

Microscopic preparations are made from selected individual colonies, which are then stained with the Gram method. Observations are carried out in a bright field in the Zeiss microscope, at a magnification of 1000 x.

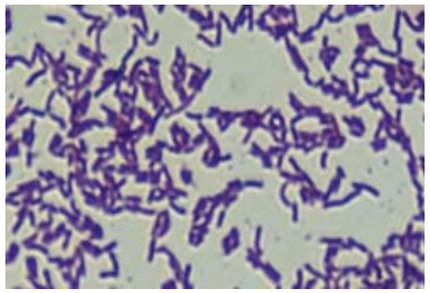
Results

Table. Microscopic evaluation of bacteria on the basis of Gram staining

Species/strain	Morphology	Arrangement	Catalase	Characteristics of colonies on solid medium
Lactobacillus rhamnosus GG	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Lactobacillus rhamnosus LR04	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Lactobacillus rhamnosus LR05	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Lactobacillus acidophilus LA1	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Lactobacillus delbrueckii spp. *bulgaricus* LB2	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Lactobacillus plantarum LP02	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Lactobacillus plantarum LPO1	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Lactobacillus fermentum LF 2	gram-positive sticks	chains	-	Milk-white, tear-shaped, grown up in the medium
Bifidobacterium breve BL10	gram-positive sticks	chains, palisades	-	Fine, round, brown-red colonies
Bifidobacterium breve Bbr8	gram-positive sticks	chains, palisades	-	Fine, round colonies, brown-red with white areola
Bifidobacterium longum BL03	gram-positive sticks	chains, palisades	-	Fine, round colonies, brown-red with white areola
Bifidobacterium animalis ssp. Lactis Bi 1 MDX	gram-positive sticks	chains, palisades	-	Fine, round colonies, brown-red with white areola
Streptococcus thermophilus Z57	gram-positive cocci	beads	-	Fine, round, white
Streptococcus thermophilus 9Y	gram-positive cocci	beads	-	Fine, round, white
Lactobacillus casei 101/37	gram-positive sticks	chains Y	-	Milk-white, tear-shaped, grown up in the medium



Growth of bacterial colonies on solid mediu a) Lactobacillus b) Bifidobacterium



Microscopic image of Lactobacillus rhamnosus GG (ATCC 53103) bacteria (magnification 1000 x)

Conclusions

- The micro-organisms in the products are characterized by the features typical of the group to which they belong.

1.3 Profile of metabolites present in our products

Each group of microorganisms has a specific profile of metabolites, which results from their metabolism. Microorganisms take nutrients from the medium in order to preserve life processes, including multiplication. Metabolizing the components of the medium they synthesize metabolites such as organic acids, alcohols or glycerol. Metabolites that are secreted outside the cells are used by microorganisms capable of producing them as part of a system protecting the cell or ensuring homeostasis. The presence of probiotic microorganism metabolites in the finished products is important for their biofunctionality and guarantees their stability and durability. The main metabolites of probiotic microorganisms are lactic, acetic and propionic acids, which are homo-and heterofermentation products. Acetic acid is characterized by the strongest properties inhibiting the growth of microorganisms. It effectively inhibits the growth of bacteria, molds and yeasts. The effect of organic acids to a large extent consists in lowering the pH of the environment to the level unfavorable for pathogens, as well as disturbance of metabolic processes occurring in the cells of undesirable microorganisms and active transport through cell membranes. Another important metabolite is diacetyl. It is a volatile, non-polar diketone resulting from pyruvate decomposition. It is produced by some strains of the genera Lactobacillus, Leuconostoc and Streptococcus. It shows bactericidal properties against some gram-negative bacteria by inactivating the metabolic pathway of arginine. Under aerobic conditions, lactic bacteria also produce hydrogen peroxide, the presence of which is the effect of flavoprotein oxidase and NADH peroxidase activity. The compound has strong antimicrobiological properties, consisting in denaturation of cell enzymes and peroxidation of membrane lipids, thus leading to disruption of cell membrane function and inhibition of many metabolic pathways. Another important metabolite is carbon dioxide, which is a by-product of lactic heterofermentation and has a bactericidal effect, especially against gram-negative bacteria. Bacteriocins are the substance of protein or peptide nature synthesized by most strains of bacteria, both gram--positive and gram-negative. Bacteriocins are synthesized in ribosomes and the bacteriocinogenic microorganisms are resistant to the substances they produce.

Methods

The quantitative and qualitative composition of probiotic products was determined by high-performance liquid chromatography (HPLC) using the Agilent Technologies 1200 series chromatograph. The chromatograph system consisted of: automatic sample feeder G1329B, double pump G1312B with refractometer detector G1362A. The separation was made on the Rezex ROA column. A total of 10 µl of the sample was applied to the column, the mobile phase was 0.005 N H2SO4. The flow rate was 0.6 mL/min at 40°C. Identification of the chemical compounds was carried out using the external standard method, measuring the area under the peaks (measurement and computer integration with the use of ChemStation for LC 3D systems, Agilent).

Results

The presence of 11 compounds showing pro-health and antiseptic properties were found in the probiotic products: valeric acid, heptanoic acid, acetic acid, benzoic acid, ethyl ester, 2-methylobytyl ester, 2-ethylhexyl ester, methyl ester, benzoic aldehyde, 1,2-propanediol and dodecalnol.

1.4 Antimicrobial activity of microorganisms and their metabolites present in products with respect to indicator microorganisms

Antagonistic activity of probiotic bacteria is closely related to the synthesis of specific products inhibiting the growth of undesirable microorganisms (e.g. organic

acids), which act synergistically. It should be noted that the antimicrobial activity of bacteria is a strain characteristic, resulting from the specific interaction between the bacterial strain and the indicator strain. The mechanism of antagonistic activity of probiotic bacteria is not well known. It is believed that it involves the change in environmental conditions under the influence of the production of organic acids and other metabolites unfavorable to the growth of undesirable microorganisms. These environmental factors include temperature, pH, composition of the medium at which the lactic bacteria produce products with antifungal and antibacterial activity.

Methods

The tests were carried out in order to determine the antagonism of products containing probiotic microorganisms with respect to indicator microorganisms, which included the preparation of indicator microorganisms and testing the activity of isolates using the well method.

Indicator microorganisms listed in the table below were used in the tests. The indicator strains were transferred to tubes containing 10 mL of broth with 2% glucose (in order to multiply the biomass). The cultures were carried out at 37°C, 24 hours. Then, in order to obtain a clear turf layer, the liquefied agar medium was supplemented with 10% (v/v) 24-hour indicator culture and poured on Petri dishes. 20 µL of the tested product was applied point-wise on the surface of solid medium vaccinated with indicator microorganisms. Incubation was performed at 37°C, 24 hours under anaerobic or relatively anaerobic conditions. Then, the diameters of the inhibition zone or the growth limitation of indicator bacteria were measured. The inhibition of growth of the indicator microorganism, demonstrated in complete transparency around the place of culture liquid application, was a sign of bactericidal activity of the tested strain. The bacteriostatic properties were determined on the basis of a decrease in the density of the turf (limiting the growth of the indicator strain).

Results

Table. Antimicrobial activity of the products with respect to indicator microorganisms

No	Indicator microorganisms	Probiotic product*	Probiotic product**
		Growth inhibition zone (mm)	
1	*Clostridium difficile* ATCC 9689	24	17
2	*Clostridium butyricum* ATTC 860	30	25
3	*Listeria monocytogenes* ATCC 7644	43	11
4	*Bacillus subtillis* ATCC 238557	24	15
5	*Enterococcus faecalis* ATTC 29212	18	6
6	*Staphylococcus aureus* ATCC 25923	27	32
7	*Staphylococcus pyrogenes* ATCC 19615	31	18
9	*Escherichia coli* ATCC 25922	38	21
10	*Klebsiella pneumoniae* ATCC 31488	13	28
11	*Proteus mirabilis* ATCC 12453	31	17
12	*Salmonella typhimurium* ATCC 14028	39	18
13	*Pseudomonas aereuginosa* ATCC 27853	0	0
14	*Enterobacter aerogenes* ATCC 13048	12	8
17	*Candida krusei* ATCC 14243	25	12
18	*Candida albicans* ATTC 10231	39	16
19	*Fusarium* sp.	21	11
20	*Alternaria* sp.	17	9

*Legend: * probiotic product containing the biomass of probiotic bacteria and their metabolites*
*** fermentation liquid devoid of probiotic bacteria biomass*

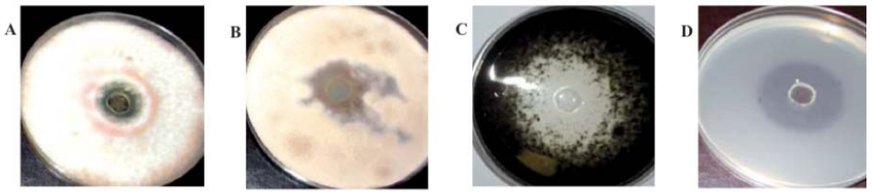


Fig. Antimicrobial effect of the probiotic product on molds of the genus Fusarium (A) and Alternaria (B) and bacteria of the genus Clostridium (C) and E. coli (D)

Conclusions

- Probiotic products show the highest antibacterial activity against *Listeria monocytogenes, Escherichia coli* and *Salmonella typhimurium*.
- Probiotic products show the highest antifungal activity against Candida albicans yeasts.
- The highest antimicrobial activity is shown by probiotic products containing the biomass of probiotic bacteria and their metabolites.

1.5 Quality stability of probiotic products during the storage

Quality and unique taste and aroma features are the main aspects through which consumers choose our products. In order to maintain the quality advantage, it is essential to maintain microbiological purity, use high quality ecological raw materials, use of unique probiotic bacteria kits and maintain appropriate quality features until the end of shelf-life. For the production of liquid probiotic products, it is essential to maintain hygienic and sanitary regimes at all stages of production and to precisely control the conditions of the production process in accordance with a unique recipe.

Each food product has a specified shelf-life. Therefore, it is important to know how the product changes and how durable it is during the storage. In the case of products containing probiotic microorganisms, the key indicator is the number of bacteria from the desired groups.

Methods

The products were stored for 12 months at room temperature (18°C) and refrigerated (4°C). Microbiological analysis was performed at the following time intervals: on production days (t0), 1 month after production (t1), 3 months after production (t3), 6 months after production (t6) and 12 months after production (t12).

Microbiological analyzes of the products were performed for: total number of psychrophilic and mesophilic microorganisms, total number of psychrophilic and mesophilic molds and yeasts, number of LAB, number of Bifidobacterium bacteria, presence and number of sporulating bacteria (including Bacillus), presence and number of anaerobic bacteria (including Clostridium), presence of Listeria monocytogenes, presence of Salmonella and presence of coliforms.

Results

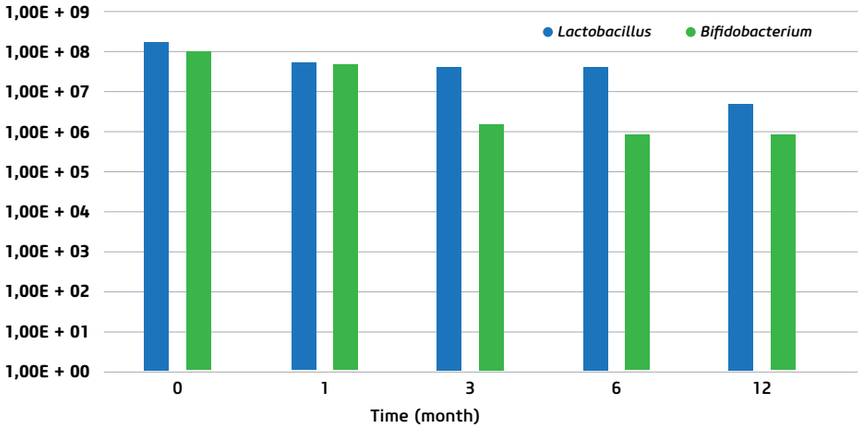


Fig. Kinetics of changes in the number of LAB contained in the probiotic product during refrigerated storage

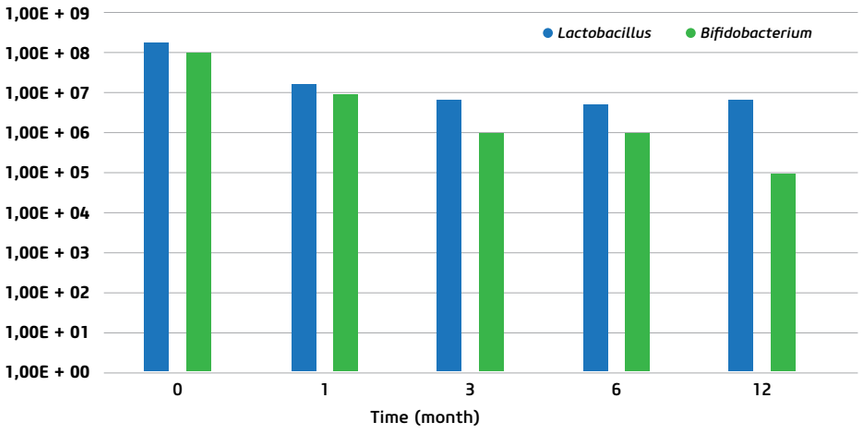


Fig. Kinetics of changes in the number of LAB contained in the probiotic product during the storage at room temperature

Conclusions

- Probiotic products are characterized by satisfactory microbiological stability during the storage.
- No Salmonella, Listeria and Bacillus microorganisms were found in the tested products (immediately after production and during the storage).
- The presence of molds was not found in the tested probiotic products.

2. Evaluation of the pro-health potential of our probiotic products

Healthy lifestyle promoted in recent years is manifested mainly through health care and physical activity. This is to ensure a longer life in optimal psychophysical condition. As a result, the demand for dietary supplements and food with the highest nutritional value and additionally with specific pro-health properties increases. Other reasons for the growing demand for food enriched with additional components aimed to improve its pro-health and taste qualities include, in particular: an aging population, increased costs of medical and social care, increased incidence of chronic diseases related to nutrition, development of knowledge on biologically active, so-called non-nutritive food ingredients and their physiological impact on the human body, increase in consumer purchasing power in developed/developing countries, development of food processing techniques and technologies, availability of new bioactive food ingredients (nutraceuticals) and the situation of the food industry in developed countries. Today's consumers are aware that not only a healthy diet but also physical activity positively affects their appearance and well-being. As a result, there is a growing demand for products that support physical performance, give energy or facilitate weight loss. In order to meet the expectations of consumers, at present many important food companies, following the developing pro-health trend, plan to introduce into their assortment products that would have a positive impact on the human body.

2.1 An effect of in vitro digestion on the number of probiotic microorganisms present in the products

One of the fundamental elements of the assessment of products biofunctionality are studies aimed at an evaluation of stability and effectiveness of active substances, mineral salts and microorganisms in conditions simulating the gastrointestinal tract. Due to difficulties in access to intestinal contents in vivo, studies on models that enable to examine digestion and absorption under *in vitro* conditions have been developed. These models are a much faster and cheaper way compared to animal testing. Scientific publications describe studies using more or less automated *in vitro* gastrointestinal models. These most advanced models of the gastrointestinal tract are computer monitored, in which, in addition to pH regulation and the addition of appropriate enzymes, peristaltic movements and absorption by the intestinal wall are imitated. There are also known studies in which digestion in the gastrointestinal tract was attempted to be simulated by creating appropriate conditions in simple laboratory vessels. Examination of bioavailability of nutrients and medicines in natural conditions is very difficult, which is to a high degree affected by the difficult access to human gastrointestinal tract lumen, especially in the small intestine section. The use of in vitro models, simulating more or less precisely the human gastrointestinal tract, affected the rapid development of scientific research. The tasks that pose a challenge for today's biotechnology concern research on the relationships between several elements applied (e.g. antibiotics, probiotic microorganisms) to the gastrointestinal tract under *in vitro* conditions. Moreover, studies on interactions between intestinal microbiome (including normal and dysfunctional) and biologically active substances under *in vitro* conditions of the gastrointestinal tract constitute an important but difficult analytical relationship.

Methods

In research on the influence of in vitro digestion on probiotic microorganisms included in our products we used the in vitro gastrointestinal tract model. The main aim of the work was to determine the kinetics of changes in the number of bacteria included in probiotic products depending on the food matrix used. The experimental system was a fermenter with a volume of 1 L (Sartorius-Poland). The temperature of the experimental system was maintained at 37°C.

The PBS buffer of pH 7.4 (control, simulation of empty stomach) and carriers in the form of food matrices: substitute milk preparation Nutramigen, used in allergy to cow's milk protein, rich in nutrients (vegetable oils, casein hydrolyzate, minerals, vitamins); unclaimed apple-carrot juice Bobo Frut-rich source of fiber (2 g/300 mL); rice glue BoboVita-starch product, rice starch is the starch most easily absorbed by the body and shows prebiotic effects, were used in the study.

Fig. Experimental system-model of in vitro digestion

First of all, a mixture of probiotic product with PBS buffer or probiotic product with selected nutritional matrix (P1)* was prepared. The test sample prepared in this way was subjected to the first stage of in vitro digestion, which was to simulate the conditions prevailing in the stomach. For this purpose, 300 U/mL of pepsin was added to the mixture imitating gastric fluid and the pH was lowered to 4.0 with 1 M HCl. The stage was carried out for 4 h at 37°C (P2). Peristaltic movements were imitated by mixing the suspension using a magnetic stirrer. The next step was to reproduce the conditions in the small intestine. For this purpose, the pH of the liquid was adjusted to 6.0 using 1 M $NaHCO_3$. Then, 10 mL of pancreatic-intestinal extract (P3) was added. The next stage involved pH increase to 7.4 by an addition of 1 M $NaHCO_3$. This stage was carried out for 2 h (P4). In order to simulate the passage of the product through the large intestine, the pH was raised to 8.0 with 2 M $NaHCO_3$. Further digestion was carried out under anaerobic conditions for 18 h (P5).

*P=measurement point

Microbiological analysis was performed using the Koch flooding method. Two groups of microorganisms were determined: LAB of the genus Lactobacillus and bacteria of the genus Bifidobacterium.

Results

In the first stage of the study, the growth of Lactobacillus and Bifidobacterium bacteria in PBS buffer was analyzed. The task of PBS buffer is to maintain the pH value at a constant level. The results of the study are presented in the table below. Initially (P1), the number of Lactobacillus was 1.9×10^8 cfu/g and Bifidobacterium 2.6×10^8 cfu/g. After 4 h incubation, Lactobacillus decreased to 2.6×10^7 cfu/g, similarly Bifidobacterium to 1.4×10^7 cfu/g. In the next stage, simulating the small intestine environment in the presence of pancreatic-intestinal juice, the number of Lactobacillus bacteria decreased again to 7.8×10^6 cfu/g. The number of Bifidobacterium at this stage was 4.7×10^7 cfu/g. At the next measurement point (P4), the number of Lactobacillus bacteria increased to 8.5×10^7 cfu/g, while the number of Bifidobacterium decreased (3.5×10^6 cfu/g). After the stage simulating the passage through the large intestine (P5), the number of Lactobacillus bacteria was similar to that at stage P4 and amounted to 9.6×10^7 cfu/g. The number of Bifidobacterium increased to 7.6×10^7 cfu/g.

Table. Kinetics of changes in the number of LAB contained in the probiotic product during the gastrointestinal passage

Measurement point	Incubation time (h)	pH	Number of Lactobacillus bacteria	*Number of Bifidobacterium bacteria*
P1	0	4.0	1.9×10^8 cfu/g	2.6×10^8 cfu/g
P2	4	4.0	2.6×10^7 cfu/g	1.4×10^7 cfu/g
P3	1.4	6.0	7.8×10^6 cfu/g	4.7×10^7 cfu/g
P4	2	7.4	8.5×10^7 cfu/g	3.5×10^6 cfu/g
P5	18	8.0	9.6×10^7 cfu/g	7.6×10^7 cfu/g

In the next stage of the study, the effect of the addition of a food matrix-Nutramigen preparation on the growth of Lactobacillus and Bifidobacterium bacteria was analyzed. The results of the study are presented in the table below. Initially (P1), the number of Lactobacillus was 1.4×10^8 cfu/g and Bifidobacterium 2.2×10^8 cfu/g. After 4 h incubation, the number of Lactobacillus bacteria increased to 7.2×10^9 cfu/g. An increase in the number of Bifidobacterium bacteria was also observed and it amounted to 3.4×10^9 cfu/g. In the next stage, simulating the small intestine environment in the presence of pancreatic-intestinal juice, the number of Lactobacillus bacteria increased again to 8.5×10^9 cfu/g. A similar observation was made for Bifidobacterium bacteria, the number of which at this stage was 5.2×10^9 cfu/g. At the next measurement point (P4), the number of Lactobacillus bacteria decreased to 9.6×10^8 cfu/g, while the number of Bifidobacterium increased to 7.7×10^9 cfu/g. At the end of the stage simulating the passage through the large intestine (P5), the number of Lactobacillus bacteria increased again to 2.6×10^9 cfu/g, while the number of Bifidobacterium remained at a level similar to that at stage P4 and amounted to 7.5×10^9 cfu/g. It can be concluded based on the study that Nutramigen provided very good conditions for growth and multiplication of both Lactobacillus and Bifidobacterium bacteria. Probably the food matrix of such a rich composition (glucose syrup, casein hydrolyzate, minerals and vitamins) also has a protective function for probiotic bacteria cells.

Table. Kinetics of changes in the number of LAB contained in the probiotic product during the gastrointestinal passage in the presence of Nutramigen preparation

Measurement point	Incubation time (h)	pH	Number of *Lactobacillus* bacteria	Number of *Bifidobacterium* bacteria
P1	0	4.0	1.4×10^8 cfu/g	2.2×10^8 cfu/g
P2	4	4.0	7.2×10^9 cfu/g	3.4×10^9 cfu/g
P3	1.4	6.0	8.5×10^9 cfu/g	5.2×10^9 cfu/g
P4	2	7.4	9.6×10^8 cfu/g	7.7×10^9 cfu/g
P5	18	8.0	2.6×10^9 cfu/g	7.5×10^9 cfu/g

In the next stage of the study, an effect of the addition of a food matrix with a high content of fiber, Bobo-Frut juice, on the growth of *Lactobacillus* and *Bifidobacterium* bacteria was analyzed. The results of the study are presented in the table below. Initially (P1) the number of *Lactobacillus* was 1.2×10^8 cfu/g and *Bifidobacterium* 5.6×10^8 cfu/g. In subsequent stages (P2, P3, P4) the number of *Lactobacillus* bacteria was similar to the initial value. An increase was observed in the last stage (P5) in which the number of Lactobacillus bacteria was 8.6×10^8 cfu/g. The number of bacteria of the genus *Bifidobacterium* was subject to more significant changes. An increase in the number of this type of bacteria was observed at the stage (P2) simulating the conditions in the stomach, where their number was 7.4×10^8 cfu/g. In the next stage, simulating the small intestine environment in the presence of pancreatic-intestinal juice, the number of *Bifidobacterium* decreased to 1.2×10^8 cfu/g. In the next measurement point (P4), the number of *Bifidobacterium* decreased and its value was close to the initial number and amounted to 5.2×10^8 cfu/g. At the end of the stage simulating the passage through the large intestine (P5), the number of *Bifidobacterium* increased again to 8.1×10^8 cfu/g. It can be concluded on the basis of the experiment that Bobo-Fru juice, used as an attractive source of fiber, provided good conditions for growth and multiplication for both *Lactobacillus* and *Bifidobacterium* bacteria. Numbers of the examined microorganisms obtained after in vitro digestion were higher than the initial values. This fact may be related to the composition of this food matrix. Fruit juices contain many substances (fiber, proteins and polyphenols) which have a protective effect on microorganisms. Moreover, fruit and vegetable juices have a buffering effect, which increases the survival of microorganisms during the passage through gastrointestinal tract.

Table. Kinetics of changes in the number of LAB contained in the probiotic product during the gastrointestinal passage in the presence of Bobo-Frut juice

Measurement point	Incubation time (h)	pH	Number of *Lactobacillus* bacteria	Number of *Bifidobacterium* bacteria
P1	0	4.0	1.2×10^8 cfu/g	5.6×10^8 cfu/g
P2	4	4.0	1.6×10^8 cfu/g	7.4×10^8 cfu/g
P3	1.4	6.0	2.0×10^8 cfu/g	1.2×10^8 cfu/g
P4	2	7.4	2.2×10^8 cfu/g	5.2×10^8 cfu/g
P5	18	8.0	8.6×10^8 cfu/g	8.1×10^8 cfu/g

In the next stage of the study, an effect of the addition of the food matrix--rice glue Bobo-Vita on the growth of *Lactobacillus* and *Bifidobacterium* bacteria was analyzed. The results of the study are presented in the table below. Initially (P1), the number of *Lactobacillus* was 3.3×10^8 cfu/g and *Bifidobacterium* 6.6×10^8 cfu/g. During the subsequent stages of the passage of microorganisms under conditions simulating the gastrointestinal tract, a similar tendency of changes in the number of *Lactobacillus* and *Bifidobacterium* bacteria was observed. In the stage simulating conditions in the stomach, the number of *Lactobacillus* bacteria decreased slightly in relation to the initial value and amounted to 2.7×10^8 cfu/g. The number of *Bifidobacterium* also decreased to 5.1×10^8 cfu/g. In subsequent stages (P3 and P4) both *Lactobacillus* and *Bifidobacterium* decreased to 1.3×10^8 cfu/g (P3), 5.5×10^7 cfu/g (P4) and 1.7×10^8 cfu/g (P3), 5.2×10^7 cfu/g (P4), respectively. An increase in the number of the examined microorganisms was observed in the last stage of the experiment (P5), in which the number of Lactobacillus bacteria was 5.8×10^8 cfu/g and the number of *Bifidobacterium* bacteria 6.1×10^8 cfu/g. It can be concluded on the basis of the experiment, that the rice glue Bobo-Vita did not provide good conditions for the growth and multiplication of *Lactobacillus* LAB and *Bifidobacterium* bacteria. The obtained numbers of the examined groups of microorganisms were higher than the initial values, but still lower compared to other food matrices. However, it is worth emphasizing the protective function of starch grains, which can be based on the surrounding of bacterial cells, thus protecting them from negative environmental impact.

Table. Kinetics of changes in the number of LAB contained in the probiotic product during the gastrointestinal passage in the presence of rice glue Bobo Vita

Measurement point	Incubation time (h)	pH	Number of *Lactobacillus* bacteria	Number of *Bifidobacterium* bacteria
P1	0	4.0	3.3×10^8cfu/g	6.6×10^8 cfu/g
P2	4	4.0	2.7×10^8 cfu/g	5.1×10^8 cfu/g
P3	1.4	6.0	1.3×10^8 cfu/g	1.7×10^8 cfu/g
P4	2	7.4	5.5×10^7 cfu/g	5.2×10^7cfu/g
P5	18	8.0	5.8×10^8 cfu/g	61×10^8 cfu/g

Conclusions

- Food matrices have a protective and prebiotic function for bacteria with probiotic potential.
- Among the tested matrices, the best conditions for the protection of viability, growth and reproduction of bacteria included in probiotic products were provided by Nutramigen, which is closely related to its rich composition.

2.2 An effect of probiotic microorganisms present in the products on normal human intestinal microbiome and dysbiotic microbiome

Abnormalities in the composition of the human gastrointestinal microbiome are closely related to lifestyle, diet and exposure to stress. The literature points to significant progress in the study of intestinal microbiome composition and its influence on autoimmune diseases, colorectal cancer, dental caries, links between the microbiome and the brain (influence on depression and autism). The influence of mother's microbiome on the settlement of the newborn organism through the placenta or even on preterm births is also studied. An important element of the study is an evaluation of the impact of environmental changes on microbiome and the development of civilization diseases, especially diabetes, obesity and cancer. In 2015, it was estimated that antibiotic-resistant pathogens cause about 50,000 deaths per year in Europe and the USA, and this will rise to 10 million deaths per year worldwide in 2050. These figures suggest that we are approaching the end of the antibiotic era. Antibiotics, especially beta-lactams and fluoroquinolones, the use of which leads to diarrhea and colitis are closely linked to the changes in the composition and function of gastrointestinal microbiome. Studies have shown that five days of fluoroquinolone administration resulted in the elimination or suppression of about one third of the microbiome in feces during three to four days of antibiotic therapy (Dethlefsen et al., 2008). Reconstruction of the population was possible within a week after the end of therapy, however, this reconstruction was incomplete (Dethlefsen and Relman, 2010). In addition, it is predicted that the etiology of 15% of cancer is related to bacterial infections. This may be due to the fact that some commensal bacteria are able to convert pro-carcinogens into DNA-damaging compounds (e.g. ethanol, heterocyclic amines) or directly produce carcinogens (e.g. fecapentaenes), as well as to stimulate the production of free oxygen radicals (e.g. Enterococcus faecalis), which increases the risk of developing colorectal cancer. There is a growing correlation between disorders in the microflora of the gastrointestinal tract and obesity. Turnbaugh et al. (2006) proved that the composition of the intestinal microflora affects body weight. The authors carried out the transfer of microorganisms from intestines of homozygous obese mice (mice with genetically determined lack of leptin resulting from nonsense mutation in 105 obesity gene codon) and mice with normal body weight to intestines of "germ free" mice (free from all detectable microorganisms and parasites). After two weeks it was observed that mice with microflora from obese mice obtained more calories from food and showed faster deposition of adipose tissue. Additionally, changes in the intestinal microbiome induce inflammation and obesity affecting epithelial cells and enteroendocrine cells and secretion of intestinal hormones: glucagon--like peptides 1 and 2 (GLP-1 and GLP-2). GLP-1 stimulates insulin secretion, delays the passage of food through the stomach, causes the symptom of satiety and weight loss, GLP-2 increases glucose transport from the intestines and decreases intestinal wall permeability. Thus, microbiome, acting on enteroendocrine cells, influences metabolism (Strober, 2013).

In addition, intestinal bacteria participate in the maturation and exchange of enterocytes, immunomodulation, gastrointestinal motor activity, metabolism of drugs, decomposition of toxins and carcinogens present in the food (e.g., heterocyclic amines, N-nitroso compounds), fermentation of undigested food components, in the production of essential vitamins (K, B12, folic acid, B1, B6), in the recirculation of bile acids (through the production of bile acids hydrolases), as well as in the protection against colonization of intestines by pathogenic bacteria, such as *Escherichia coli*, *Vibrio cholerae, Clostridium* spp., *Salmonella* spp. and *Shigella* spp.

Methods

In the first stage of the study, the variability was determined for selected groups of microorganisms included in fecal microflora of healthy, physically active people, declaring that they did not take antibiotics (for the period of min. 1 year), eating rationally and in accordance with the basic principles of dietetics, not consuming alcohol (control test); people over 75 years of age; patients 2 weeks after the end of antibiotic therapy; patients after the end of chemotherapy and obese people (BMI>30) and a standardized inoculum simulating intestinal microbiome was prepared.

The standardized intestinal microbial inoculum was derived from donors from the above mentioned groups and contained probiotic bacteria (*Lactobacillus* and *Bifidobacterium* genera), immunity stimulating bacteria (non-pathogenic *E. coli* and *Enterococcus* genera) as well as potentially pathogenic microflora (proteolytic bacteria, *Clostridium* genus bacteria and yeast-like fungi of *Candida genus*). The presence and abundance of microorganisms from a given group varied depending on the origin of the inoculum. Microbiological media suitable for the given type were used to isolate and determine the number of microorganisms.

Analyzed components of human intestinal microbiome

Undesirable microorganisms	Desirable microorganisms
- *Lactobacillus* bacteria	- proteolytic bacteria
- *Bifidobacterium* bacteria	- lactoze negative *E.coli* bacteria
- Nonpathogenic bacteria *coli*	- *Clostridium* bacteria
- *Enterococcus* sp. bacteria	- *Candida* yeast

Results

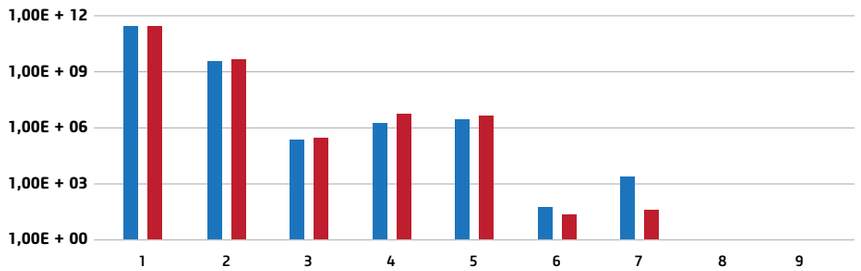


Fig. An effect of probiotic product on qualitative and quantitative changes of intestinal microbiome in healthy subjects

Legend: blue bars-microbial abundance in a standardized intestinal microbial inoculum; red bars-microbial abundance in a standardized intestinal microbial inoculum after in vitro digestion in the presence of a probiotic product

1- total number of microorganisms; 2- *Bifidobacterium*; 3- *Lactobacillus*; 4- nonpathogenic *E.coli*; 5- *Enterococcus*; 6- *Clostridium*; 7- *E.coli*; 8- proteolytic bacteria; 9-Yeast

Conclusions

- The presence of organic acids in the probiotic product resulted in a significant reduction of undesirable microorganisms of the genera *Clostridium* and *E. coli.*
- Microorganisms from other groups remained at a similar level compared to inoculum, which may indicate that the microflora from healthy individuals has high resistance to organic acids and is characterized by good vitality.

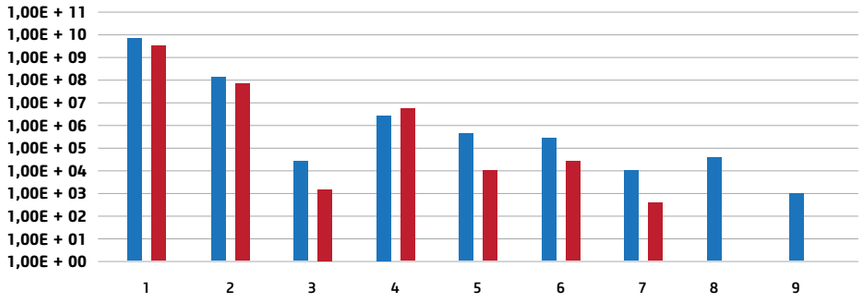


Fig. An effect of probiotic product on qualitative and quantitative changes of intestinal microbiome in elderly subjects

Legend: blue bars-microbial abundance in a standardized intestinal microbial inoculum; red bars-microbial abundance in a standardized intestinal microbial inoculum after in vitro digestion in the presence of a probiotic product

1- total number of microorganisms; 2- *Bifidobacterium*; 3- *Lactobacillus*; 4- nonpathogenic E.coli; 5- *Enterococcus*; 6- *Clostridium*; 7- *E.coli*; 8- proteolytic bacteria; 9-Yeast

Conclusions

- The reduction in the majority of groups of microorganisms determined in the composition of the intestinal microbiome was observed after the application of the probiotic product.
- Moreover, total elimination of undesirable microorganisms, i.e., proteolytic bacteria and yeast-like fungi, was observed.
- The microorganisms in the intestinal microbiome of the elderly may be characterized by reduced vitality and increased sensitivity to organic acids and polyphenols.

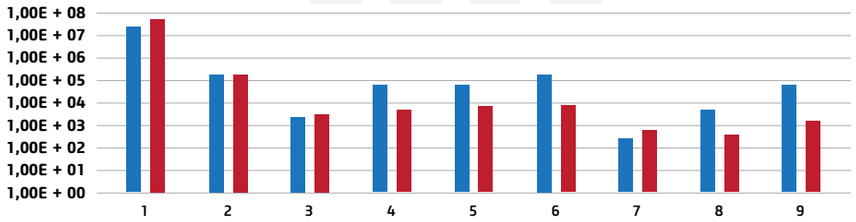


Fig. An effect of probiotic product on qualitative and quantitative changes of intestinal microbiome in subjects after completed antibiotics therapy

Legend: blue bars-microbial abundance in a standardized intestinal microbial inoculum; red bars-microbial abundance in a standardized intestinal microbial inoculum after in vitro digestion in the presence of a probiotic product

1- total number of microorganisms; 2- *Bifidobacterium*; 3- *Lactobacillus*; 4- nonpathogenic *E.coli*; 5- *Enterococcus*; 6- *Clostridium*; 7- *E.coli*; 8- proteolytic bacteria; 9-Yeast

Conclusions

- An increase in the number of microorganisms with probiotic potential was observed after an application of the probiotic product.
- Moreover, the reduction of undesirable microorganisms such as *Clostridium* bacteria, proteolytic bacteria as well as yeast-like fungi was observed.

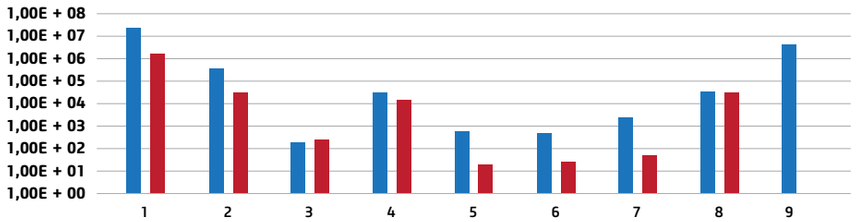


Fig. An effect of probiotic product on qualitative and quantitative changes of intestinal microbiome in subjects treated with chemotherapeutics

Legend: blue bars-microbial abundance in a standardized intestinal microbial inoculum; red bars-microbial abundance in a standardized intestinal microbial inoculum after in vitro digestion in the presence of a probiotic product

1- total number of microorganisms; 2- *Bifidobacterium*; 3- *Lactobacillus*; 4- nonpathogenic *E.coli*; 5- *Enterococcus*; 6- *Clostridium*; 7- *E.coli*; 8- proteolytic bacteria; 9-Yeast

Conclusions

- An application of the probiotic product resulted in the complete elimination of yeast-like fungi.
- In addition, a reduction in the number of most microbial groups was observed, which may indicate a strong weakening of microbiome vitality, which was exposed to toxic substances (chemotherapeutics).

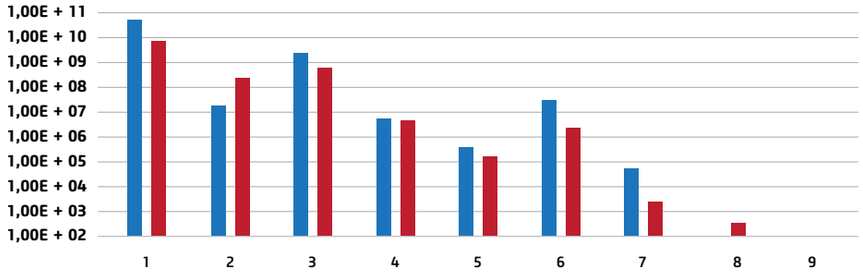


Fig. An effect of probiotic product on qualitative and quantitative changes of intestinal microbiome in obese people

Legend: blue bars-microbial abundance in a standardized intestinal microbial inoculum; red bars-microbial abundance in a standardized intestinal microbial inoculum after in vitro digestion in the presence of a probiotic product 1- total number of microorganisms; 2- Bifidobacterium; 3- Lactobacillus; 4- nonpathogenic E.coli; 5- Enterococcus; 6- Clostridium; 7- E.coli; 8- proteolytic bacteria

Conclusions

- An application of the probiotic product resulted in a reduction of all microbial groups except for *Bifidobacterium* and proteolytic bacteria.
- An increase in the number of *Bifidobacterium* bacteria is beneficial since it leads to a qualitative and quantitative equilibrium between the Firmicutes group (*Lactobacillus* bacteria) and *Bifidobacteriales.*

2. Evaluation of the ability of probiotic microorganisms present in the products to adhere to epithelial cells in the in vitro model-tests on cell lines

The ability of probiotic microorganisms to adhere to intestinal epithelium cells is one of the key features of microorganisms required for their subsequent pro-health effects on the human body. It enables direct contact of probiotic

bacteria with host cells, and also prolongs their residence time in the digestive tract. Therefore, in addition to studies on sensitivity to the influence of low pH, or the presence of bile salts, as well as digestive enzymes, it is one of the basic indicators of probiotic preparations effectiveness.

Methods

In addition to probiotic products, CaCo-2 cell line isolated from human colorectal cancer cells and HT-29 line from colorectal adenocarcinoma were used in the study. Both lines originated from the American Collection of Cellular Cultures (ATCC). CaCo-2 and HT-29 intestinal epithelium cells were cultured in DMEM medium, containing the addition of essential amino acids and gentamicin. Firstly, the appropriate volume of cell suspension, which is a probiotic product containing 6.0×10^8 cfu/mL, was collected. The whole was supplemented with DMEM medium and antibiotic. The culture of CaCo-2 and HT-29 cells was carried out in an incubator at 37°C and a gas atmosphere containing 5% CO2 and 95% air, with medium exchange every 24 hours. The culture was carried out for 21 days for Caco-2 culture and 14 days for HT-29. The number of CaCo-2 and HT-29 cells was determined under the microscope. Before the test, CaCo-2 and HT-29 cells were washed with PBS solution, and DMEM medium containing probiotic bacteria cells was introduced. The cultures were incubated for 2 hours at 37°C. Then Triton X-100 solution was added to each well and a lysis of CaCo-2 and HT-29 cells was carried out. The lysates were transferred to Eppendorff tubes and centrifuged. The supernatant was removed and the precipitate was suspended in saline. The inoculations from selected dilutions were performed in three parallel repetitions. The cultures were carried out in MRS medium with 2% agar at 35°C for 48–72 h.

Results

For the determination of adhesion properties of probiotic strains included in probiotic products, 21-day CaCo-2 cell culture and 14-day culture of epithelial cell HT-29 were used. These are the cell models most frequently used for the determination of adhesion capacity of microorganisms. After the intestinal passage, the number of bacterial cells associated with intestinal epithelium cells was determined. The adhesion capacity of probiotic bacteria was expressed as the level of adhesion, i.e., the number of microbial cells associated with 100 CaCo-2 and HT-29 cells.

The table below presents the results of adhesion ability of probiotic microorganisms in the product after digestion in the small intestine. The obtained results indicate the highest affinity to epithelial cells of bacterial preparation with the addition of food matrix in the form of substitute milk. The number of bacteria associated with 100 intestinal epithelial cells was 258 for CaCo-2 and 409 for HT-29, respectively. The lowest number of adhered cells, as in the case of CaCo-2 cells analysis, was observed for the product without the use of food matrix. In the latter variant, unlike the others (using a food matrix) both with CaCo-2 and HT-29 lines, the number of adhered cells decreased after an incubation under conditions imitating the digestion process in the small intestine. Comparing also the number of bacteria associated with 100 CaCo-2 and HT-29 cells, it can be stated that the cells included in the probiotic product have a higher affinity to HT-29 cells. Comparing samples with the highest number of bacterial cells adhering to CaCo-2 and HT-29 cells, it can be observed that about 100 more cells undergo the process of adhesion to HT-29 cells.

Table. Number of probiotic bacteria associated with 100 HT-29 and CaCo-2 cells after in vitro digestion process

Cell line Adhesion index	CaCo-2 Number of related bacteria/100 CaCo-2	HT-29 Number of related bacteria/100 HT-29
Probiotic product **- without food matrix addition**	110	201
Probiotic product with food matrix addition - **substitute milk**	258	409
Probiotic product with food matrix addition - **Bobo Frut**	210	293
Probiotic product with food matrix addition - **rice glue**	223	354

The same conclusions can be drawn from the percentage adhesion degree determined at the beginning of the analysis, as shown in the figure below. This parameter describes the ratio of the number of bacterial cells that adhered to the number of all cells introduced. The obtained results of the degree of adhesion also indicate that the probiotic bacteria included in the product have a high adhesion capacity to model epithelial cells of the HT-29 line. This value depends on the food matrix used in the experiment. Clearly, the highest effectiveness of adhesion, similarly to the results obtained after the digestion process, can be observed in the sample, which was enriched with food matrix in the form of substitute milk. The lowest degree of adhesion to HT-29 cells is visible in the variant in which the preparation was subjected to experiments without food matrix. The degree of bacterial cells adhesion to CaCo-2 line remains at a low level of about 6% in all four examined variants.

Conclusions

The highest level of microorganisms adhesion with the addition of a food matrix in the form of milk powder is related to the presence of a number of protective substances in this product. As already presented in another report, milk replacers are rich in many protective substances, including polyols, disaccharides, polysaccharides, amino acids, as well as protein hydrolyzates, minerals, salts of organic acids and many others. In this study, the most effective protection of adhesion properties was demonstrated by substitute milk, where the number of bacteria subject to adhesion to CaCo-2 and HT-29 cells was the highest. It is worth mentioning that milk is a commonly used substance to protect the survival and functional properties of bacterial cells. It is a natural environment for LAB, and due to the content of proteins, numerous vitamins and minerals, and above all lactose as a substrate, it is a growth medium for these microorganisms. Lactose, creating hydrogen bonds with cell membrane proteins, also increases its stability. Due to its buffer capacity, milk also reduces the acidic reaction of the stomach environment, thus contributing to a reduction in mortality during the intestinal passage.

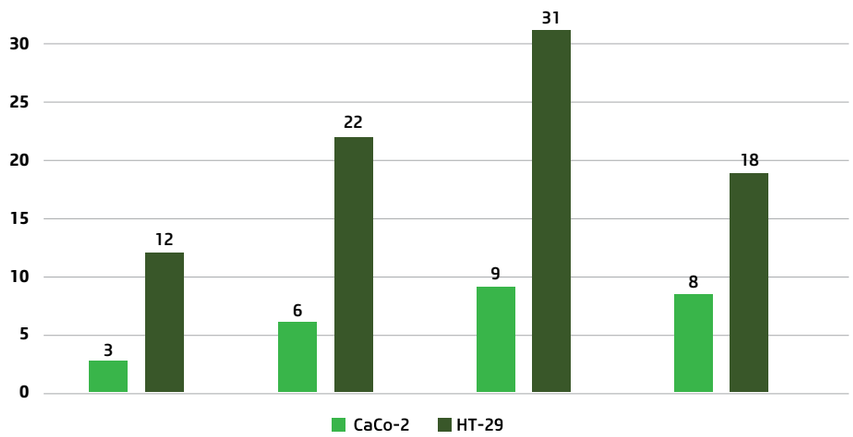


Fig. Degree of adhesion of undigested probiotic bacteria before the intestinal passage (%)

OUR COMPANY

Living Food Sp. z o.o., Graniczna 15, Trzciel, Poland

Fig. View of the part of the laboratory and the production line

PHOTO-RELATION

Our company has its headquarters in a building with an area of about 500 m^2 which has been divided into three main parts. The first one is the office part, occupying the smallest space and performing an administrative function. The second part is the laboratory part, where the space is divided into positions for bio-processes conducting on a semi-industrial scale, analytical stands, an incubator and a technical room. The third is technological part. The largest surface area of the company consists of five rooms, which are used for pre-treatment and preparation of raw materials, sterilization processes, preparation of microbial starter cultures, propagation and fermentation processes, separation processes, packaging, labeling and intermediate storage. The finished products warehouse is located in Trzciel, at the facility at Pl. Zjednoczenia Narodowego 13.

AWARDS

Our products have numerous awards, including:

- Prof. Zbigniew Religa Cardiac Surgery Development Foundation in Zabrze
- Certificate awarded for the first position in the “Nasze Dobre Lubuskie” (Our Good Lubuskie) competition of food products
- Certificate awarded by the Wielkopolska Institute of Quality for the improvement of our products in the scope of quality within the project “Creation and implementation of pro-innovative optimization services for SMEs based on an integrated expert system”
- Certificate of the nationwide Promotion Programme “Doceń Polskie” (Appreciate Polish)
- First degree distinction in the 16th edition of the „Wielkopolska Nagroda Jakości” (Wielkopolska Quality Award) competition for the implementation of the quality management concept
- Polish Innovation Award 2018 awarded by the Polish Agency for Entrepreneurship
- Title and award “Agribusiness Eagle 2018” granted by EMS Promotion and Publishing Agency

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