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Review

Probiotics, prebiotics and antioxidants as functional foods*

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The term "functional foods" comprises some bacterial strains and products of plant and animal origin containing physiologically active compounds beneficial for human health and reducing the risk of chronic diseases. Among the best known functional compounds probiotics, prebiotics and natural antioxidants should be given as examples. These substances can be obtained by biotechnological methods and by extraction from plant or animal tissues.

Keywords: probiotics, prebiotics, antioxidants, functional food

Within the last decade, we have observed distinct changes in the understanding of the role of foods in human health promotion. The first frontier of scientific investigations has moved from the primary role of food as the source of energy and body-forming substances to the more subtle action of biologically active food components on human health. In the industrialized world, there has been an explosion of consumer interest in the active role of foods in the well-being and life prolongation as well as in the prevention of initiation, promotion and development of cancer, cardiovascular diseases and osteoporosis. As a result, a new term — functional food — was proposed (Berner & O'Donnell, 1998; Dimer & Gibson, 1998; Sanders, 1998; Diplock *et al.*, 1999; Pisulewski & Kostogrys, 2003).

According to the definition, functional food is a part of an everyday diet and is demonstrated to offer health benefits and to reduce the risk of chronic disease beyond the widely accepted nutritional effects. The term 'functional foods' was introduced in Japan in mid 1980s. This type of foods is known on the Japanese market as "Foods for Specified Health Use" (FOSHU). The functional foods comprise: (i) conventional foods containing naturally occurring bioactive substances (e.g., dietary fiber), (ii) foods enriched with bioactive substances (e.g., probiotics, antioxidants), and (iii) synthesized food ingredients introduced to traditional foods (e.g., prebiotics). Among the functional components, probiotics and prebiotics, soluble fiber, omega-3 – polyunsaturated fatty acids, conjugated linoleic acid, plant antioxidants, vitamins and minerals, some proteins, peptides and amino acids, as well as phospholipids are frequently mentioned. These active substances constitute a focus of contemporary science of human nutrition. A wide range of food products offer a variety of physiologically active compounds; functional food should be understood as a new idea, rather than a defined product. It should be also stressed that functional foods are not pills or capsules but are consumed as part of a normal everyday diet. Epidemiological studies and randomized clinical trials carried out in different countries have demonstrated or at least suggested numerous health effects related to functional food consumption, such as reduction of cancer risk, improvement of heart health, stimulation of immune system, decrease of menopause symptoms, improvement of gastrointestinal health, maintenance of urinary tract health, anti-inflammatory effects, reduction of blood pressure, maintenance of vision, antibacterial and antiviral activities, reduction of osteoporosis and anti-obese effects. These effects can be claimed on food product labels, a practice more and more commonly introduced for the promotion of functional foods in the food market.

At the moment, the most important and the most frequently used functional food compounds are probiotics, prebiotics, plant antioxidants, vitamins and calcium. It has been given high priority in the production of probiotics and prebiotics, and

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the extraction of bioactive components from plant materials by enzyme and fermentation technology to reduce loss of these compounds as well as by genetic engineering to intensify their biosynthesis. At the present time, biotechnology plays a key role in the functional food industry. However, transgenic foods are not well accepted in the European Union and food industry companies prefer to employ methods of conventional biotechnology.

PROBIOTICS

Probiotics are defined as selected, viable microbial dietary supplements that, when introduced in sufficient quantities, beneficially affect human organism through their effects in the intestinal tract (Dimer & Gibson, 1998; Zimmer & Gibson, 1998; Sanders, 1998; Vaughan *et al.*, 1999; Zubillaga *et al.*, 2001; Holzapfel & Schillinger, 2002). Also FAO/WHO has adopted the definition of probiotics as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). There are a large number of probiotics currently used and available in dairy fermented foods, especially in yogurts. Lactic acid bacteria constitute a diverse group of organisms providing considerable benefits to humankind, some as natural inhabitants of the intestinal tract and others as fermentative lactic acid bacteria used in food industry, imparting flavor, texture and possessing preservative properties. Beyond these, some species are administered to humans as live microbial supplements, which positively influence our health mainly by improving the composition of intestinal microbiota. For this reason, they are called probiotics. Some selected strains of *Lactobacillus, Bifidobacterium, Streptococcus, Lactococcus* and *Saccharomyces* have been promoted in food products because of their reputed health benefits (Dimer & Gibson 1998; Sanders, 1998; Fuller, 1991; Ouwehand *et al.,* 1999; Puupponen-Pimia *et al.,* 2002).

The physiological effects related to probiotic bacteria include the reduction of gut pH, production of some digestive enzymes and vitamins, production of antibacterial substances, e.g., organic acids, bacteriocins, hydrogen peroxide, diacetyl, acetaldehyde, lactoperoxidase system, lactones and other unidentified substances, reconstruction of normal intestinal microflora after disorders caused by diarrhoeas, antibiotic therapy and radiotherapy, reduction of cholesterol level in the blood, stimulation of immune functions, suppression of bacterial infections, removal of carcinogens, improvement of calcium absorption as well as the reduction of faecal enzyme activity (Ouwehand *et al.,* 1999; Zubillaga *et al.,* 2001; Holzapfel & Schilling 2002).

To achieve a probiotic status, microorganisms must fulfill a number of criteria related to safety, functional effects and technological properties (FAO/WHO, 2001).

From the safety point of view, the probiotic microorganisms should not be pathogenic, have no connection with diarrhoeagenic bacteria and no ability to transfer antibiotic resistance genes, as well as be able to maintain genetic stability. To be recognized as functional food components, they should demonstrate the following properties: acid- and bile-stability, resistance to digestive enzymes, adhesion to intestine surface, antagonistic activity against human pathogens, anti-carcinogenic and anti-mutagenic activity, cholesterol-lowering effects, stimulation of the immune system without inflammatory effects, enhancement of bowel motility, maintenance of mucosal integrity, improvement of bioavailability of food compounds and production of vitamins and enzymes (Ouwehand *et al.*, 1999).

The technological properties of bacteria play a very significant role in the production of probiotics (Saarela *et al.*, 2000). They possess good sensorial properties, fermentative activity, good survival during freeze-drying or spray-drying, proper growth and viability in food products, phage resistance and high stability during long-term storage.

The majority of bacteria belonging to the *Lactobacillus* and *Bifidobacterium* genera are recognized as safety. It is generally accepted that, with the only exception of streptococci and enterococci, lactic acid bacteria are rarely pathogenic to humans and animals. They have been used in production of foods since ancient times with no negative effects on humans. However, the list of probiotic strains is rather short. It includes strains offered by the dairy industry and some scientific groups (Ouwehand *et al.*, 1999; Holzapfel & Schillinger, 2002).

The probiotic properties of probiotic bacteria are usually studied using different models, as follows:

1, *in vitro* epithelial cell cultures;
2, laboratory animals (mouse and rat);
3, human volunteers.

Investigations performed with human volunteers in clinical trials are generally accepted as having the highest scientific value. However, such investigations are only rarely possible. At present, the commonly used models include epithelium cell cultures and laboratory animals.

The *in vitro* epithelial cell cultures demonstrate morphological and physiological similarities to the enterocytes *in vivo*. The most often used cell lines are Caco-2 and HT-29 (Fig. 1). Caco-2 cells were isolated from a neoplastic tumor of the human large intestine and present the morphology of small intestine enterocyte-like cells. The HT-29 cell line is

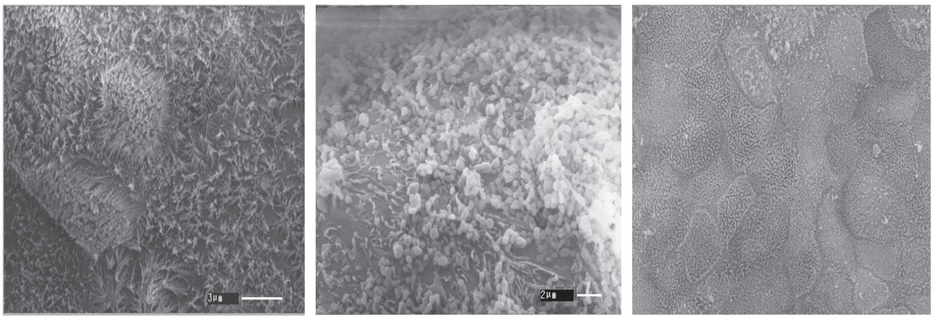

Figure 1. Morphology of cell monolayer of well-developed Caco-2, HT-29 and Int 407 cultures, respectively.
The cultures were grown in Dulbecco's modified Eagle medium at 37°C in a 5% CO_2/95% air atmosphere and were 21 days old (Lewandowska *et al.*, 2005).

very similar to the large intestine cells and contains globlet-like cells. They produce mucin on their cell surface. The both lines are applied to bacterial adhesion examination and in assay of their biological activities, e.g., the immune response of epithelial cells to probiotic bacteria (Grajek & Olejnik, 2004). Investigations on bacteria adherence to epithelial cells *in vitro* are expensive, therefore, new culture methods as well as simple *in vitro* models have been proposed (Olejnik *et al.*, 2003).

In the literature, the use of different solid surface models, such as mucosa, alginate, carrageenan, gelatin, collagen, glass, polystyrene and carboxymethylcellulose are also described (An & Friedman, 1997). However, numerous investigations have shown that none of the simple models exhibit comparable adhesion properties to those presented by epithelial cell cultures.

It should be stressed that the results obtained with the *in vitro* models are not sufficient and require confirmation in double blind, randomized, placebo-controlled human trials.

From the practical point of view, the technological aspects of probiotic production also play a very important role. During the technological processing bacteria cells are exposed to different stresses (Knorr 1998; Mattila-Sandholm *et al*., 2002). In a bioreactor, medium stirring causes mechanical stress, high liquid volume causes hydrostatic pressure, bacteria growth is connected with quick nutrient depletion and accumulation of harmful metabolites in the bacterial culture. During separation, bacteria cells are exposed to mechanical stress caused by pumps, centrifuges and membrane filters which considerably reduces cell structure integrity. Also negative effects on cell viability are exerted by the extreme temperature conditions during heat-drying or freeze-drying (Desmond *et al*., 2001). Additionally, cell dehydration is accompanied by a significant increase in the intracellular osmotic pressure, again a strong detrimental factor. As a result, during downstream processing the cell viability decreases and alterations in cell metabolism are observed. Quite contrarily, the market requires probiotic cultures with high viability and fermentative activity. The accepted living cell density is over 10^6 cfu/ml. To resolve these problems intensive research is carried out in many laboratories all over the world (Knorr 1998; Mattila-Sandholm *et al*., 2002). These investigations have created a scientific basis and practical experience to develop the technology of lactic acid bacteria starter cultures and production of some bacterial metabolites.

PREBIOTICS

Prebiotics are an alternative for probiotics or their cofactors. They are defined as non-digestible or low-digestible food ingredients that benefit the host organism by selectively stimulating the growth or activity of one or a limited number of probiotic bacteria in the colon (Crittenden & Playne, 1996; Dimer & Gibson, 1998; Zimmer & Gibson, 1998; Manning & Gibson, 2004). This role is played by fermentable carbohydrates, which are not digested or poorly digested in the small intestine and stimulate, preferentially, the growth of bifidobacteria and some Gram-positive bacteria, belonging to the probiotic bacteria administered to humans. Complex carbohydrates pass through the small intestine to the lower gut where they become available for some colonic bacteria but are not utilized by the majority of the bacteria present in the colon. Lactulose, galactooligosaccharides, fructooligosaccharides, inulin and its hydrolysates, maltooligosaccharides, and resistant starch are prebiotics commonly used in human nutrition. The main end products of carbohydrate metabolism are short-chained fatty acids, namely acetate, butyrate and propionate, which are further used by the host organism as an energy source.

In practice, the most common oligosaccharides are inulin and its hydrolysates and oligofructans. They can be found in chicory, topinambuco, onion, garlic, asparagus, artichoke, leek, bananas, tomatos and many other plants.

Oligosaccharides comprise glycosides that contain between three and ten sugar moieties. However, disaccharides are also included in this group. The degree of oligosaccharide polymerization is of importance. Usually, food-grade oligosaccharides are mixtures of saccharides with a different degree of polymerization (Crittenden & Playne, 1996). For instance, the major fraction in inulin has a degree of polymerization of about 14.

Prebiotic oligosaccharides can be produced in three different ways: by extraction from plant materials, microbiological synthesis or enzymatic synthesis, and enzymatic hydrolysis of polysaccharides (Crittenden & Playne, 1996; Gulewicz *et al.,* 2003). The majority of prebiotic oligosaccharides are produced on the industrial scale and are widely available on the market.

Recently, many patents concerning prebiotic oligosaccharides have been claimed and this field is continuously increasing (Crittenden & Playne, 1996).

In practice, combined mixtures of probiotics and prebiotics are often used because their synergic effects are conferred onto food products. For this reason, such mixtures are called synbiotics.

ANTIOXIDANTS

Plant antioxidants constitute one of the most active food compounds (Diplock *et al.*, 1996; Peterson & Dwyer, 1998; Surh, 1999; Kris-Etherton *et al.,* 2002; Schieber *et al.,* 2001). The main source of these substances is plant material. Garlic, broccoli, green tea, soybean, tomato, carrot, Brussels sprouts, kale, cabbage, onions, cauliflower, red beets, cranberries, cocoa, blackberry, blueberry, red grapes, prunes, and citrus fruits are mentioned as the richest sources of antioxidants. The content of phenolic antioxidants calculated per one kilogram of plant dry matter amounts to from about 0.1–1.0 g in the majority of fruits and vegetables up to 226 g in green leaves of tea (King & Young, 1999).

The chemical classification of antioxidants is very complicated. According to the mode of action, two main groups of antioxidants can be distinguished. The first comprises the chemical substances which interrupt the propagation of the free radical chain by hydrogen donation to radicals or stabilization of relocated radical electrons. Such mode of action is demonstrated by tocopherols, gallusans, and hydrochinons. The second group is characterized by a synergistic mode of action. It includes oxygen scavengers and chelators which bind ions involved in free radical formation. Their activity consists in hydrogen delivery to phenoxyradicals that leads to the reconstitution of the primary function of antioxidants. This role is played by substances binding metal ions, e.g. citric acid, and by secondary antioxidants, such as amino acids, flavonoids, β-caroten, selenium and many others.

Free radicals are a major cause of many degenerative diseases, such as atherosclerosis, cancer, cardiovascular diseases, inflammatory bowel diseases, skin aging, old age dementia and arthritis. Epidemiological data and randomized clinical trials provide ample indications that antioxidants play a fundamental role in the prevention of cancer and cardiovascular diseases (Shklar, 1998; Surh, 1999; Kris-Etherton *et al.,* 2002; Ferrari & Torres, 2003). They act as scavengers of reactive oxygen species and metal chelators that protect human cells and reduce oxidative damages.

An important role in functional food evaluation is played by the bioavailability of antioxidants, which is considered as a key-factor in the biological activity of substances in the alimentary tract and their absorption through the intestinal walls into the blood circulation system. This research is very difficult to conduct as it is impossible to monitor the interior of the intestines and, at the same time, apply a very complicated procedure of taking samples for further analysis. Consequently, there is a need for the development of models in '*in vitro*' research, which would mimic the conditions of the alimentary tract. For the assessment of the antioxidant uptake, the '*in vitro*' models of human epithelial cell cultures are commonly applied.

One of the main causes of cancerogenesis initiation is DNA damage provoked by mutagenic factors (genotoxic), e.g. free radicals (Diplock *et al.,* 1999). The key role in the cancerogenic transformation of cells is played by mutations in genes which control cell growth and differentiation and participate in combining the extracellular and intracellular signalization with the cell response. Mutations in these genes may lead to the initiation, promotion and progress of cancer. It has been demonstrated, on the basis of epidemiological studies, that taking large quantities of antioxidants may significantly reduce the risk of cancer diseases. Factors inhibiting the promotion and progress of cancer include: β-carotene, curcumin, gingerol, gallusan, epigallocatechine, and resveratrol (Shklar, 1998).

The development of existing tumors is also significantly inhibited by antioxidants. At the moment, three main mechanisms of inhibition and destruction are postulated. The first of them consists in increasing the immunological activity of the organism, which leads to a better identification of cancer cells and their destruction. The extermination factors include the tumour necrosis factor α (TNF-α) manufactured by macrophages and β tumour necrosis factor β (TNF-β) produced by lymphocytes. It has been demonstrated that antioxidants, such as α-tocopherol and β-carotene increase the production of cytotoxic cells, activate them for the production of large quantities of cytokines and facilitate their migration to cancer cells. This results in the destruction of the proliferating form of the tumor. The second mechanism of tumor eradication is of genetic nature and here, too, antioxidants take an active part. Their role consists in enhancing the expression of the wild type of *p53* gene whose product has the nature of a suppressive protein and in decreasing the expression of *p53* mutants acting as oncogenes. It was also demonstrated that β-carotene is capable of suppressing other oncogenes (MacPhee, 1998; Ferguson, 2001).

It should be strongly emphasized here that the action of antioxidants depends on the dose taken (Stahl *et al.*, 2002). At too high doses, their role changes and instead of acting as protective substances, they themselves become pro-oxidants and result in very dangerous disease symptoms.

The impact of food components on the human organism functions is assessed with the assistance of biomarkers (Diplock *et al.*, 1999). This notion comprises factors or indicators representing the course of a given biological process. Depending on the process examined, they may be of biochemical, physiological or behavioral nature. The determination of biomarkers should be easy to perform, reproducible, unambiguous, sensitive and specific. Surrogate endpoint biomarkers (SEBs) are widely employed for the assessment of the protective activity of antioxidants against cancerous or blood circulation system diseases in short-term examinations (Einspahr *et al.*, 1997).

Biomarkers for the identification of oxidation processes associated with diseases of the blood circulation system have also been developed. They apply to the oxidation of lipoproteins in the bloodstream and unsaturated fatty acids in membrane phospholipids. The oxidation of LDL is particularly important in this regard as it leads to atherosclerotic changes (Gey, 1995). Assays of the levels of lipid hydroperoxides or their derivatives (e.g., malonedialdehyde) or isoprostanes are used as biomarkers (Griffith *et al.*, 2002).

At present, we can observe the beginnings of a new discipline – nutrigenomics – which puts forward a totally new approach to the monitoring of biological phenomena in the human organism, associated with nutrition (Roberts *et al.*, 2001; van Ommen & Stierum, 2002). This new approach consists in analysis of many minute, even discrete changes associated with the genetic response to nutritive stimuli in place of an analysis of reactions associated with the expression of single genes, in much the same way it is done in the research on the effects of drugs. This kind of approach requires prior knowledge of unknown biochemical and physiological effects, which are difficult to identify with the help of the developed markers (a single gene, a single protein or a single metabolite). This situation is further confounded by the fact that individual dietary functional constituents occur in a cocktail with other substances. In addition, the entire organism is in a state of homeostasis, which consists in maintaining a chemical balance between mutually counteracting and opposing metabolic reaction. The key role is played by arresting and integrating changes associated with a given disease (or a physiological phenomenon) at all levels of molecular information (mRNA, protein, metabolite). However, it should be emphasized that these types of markers are only at the initial stage of investigations.

The concentration of antioxidants in raw materials depends, to a considerable extent, on the variety of crop plants and conditions of their cultivation. In addition, also chemical changes and quantitative losses of selected groups of antioxidants and their biological activities in the entire processing chain starting with the raw material, through technological treatment, storage and culinary preparation until their absorption in the alimentary tract, all play an important role in the assessment of the antioxidant activity.

Antioxidants found in food raw materials undergo similar changes during technological treatments as other components (Pokorny & Schmidt, 2001). The literature on the subject is somewhat limited, as it is concerned, primarily, with losses of individual antioxidants in the course of single processes without taking into consideration changes in their biological activities. Many of the reported investigations are based on model experiments in which synthetic antioxidants were introduced into products. The technological processing is frequently based on the application of drastic procedures, which include a wide range of thermal or hydrothermal processes such as: pasteurization, sterilization, blanching, thickening by evaporation as well as drying, extrusion and microwave heating and such culinary treatments as cooking, frying, stewing and roasting. The new processing methods, such as pascalization and treatment with electromagnetic field should also be considered as extreme. Changes in the bioactive constituents can be caused by the microbiological and enzymatic processes taking place during fermentation. Apart from temperature and pressure, also the contact of molecules with oxygen and light can affect the chemical stability of components. This is strongly influenced by new methods of food packaging and conditions of its storage. Therefore, the maintenance of a high antioxidizing activity of antioxidants is a complex issue, frequently difficult to analyze. Tomas-Barberan and Espin (2001) presented a comprehensive study devoted to changes of phenol compounds depending on the type of treatment and storage conditions of fruit and vegetables.

However, it is their bioavailability that is believed to play the fundamental role in the biological activity of antioxidants (Stahl *et al.*, 2002). This concept is defined in several different ways, nevertheless, its essence can be reduced to the question: what part of the active substance introduced *per os* becomes digested, absorbed and incorporated into normal metabolic processes. The matter is poorly recognized and requires much more attention.

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