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EPITHELIAL CELL CULTURES *IN VITRO* AS A MODEL TO STUDY FUNCTIONAL PROPERTIES OF FOOD

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This review presents the applications of the *in vitro* cultures of human epithelial cells as a model for the study on functional properties of probiotic bacteria and bioactive food compounds. The main cell lines used in the research are Caco-2 as a model of small intestine and HT-29, as a model of large intestine. The structure, monolayer formation and physiology of epithelial cells are described. The commonly used growth media, culture vessels and growth conditions of the *in vitro* epithelial cultures are discussed. One of the most important applications of epithelial cell cultures are the studies on adhesion of probiotic bacteria and invasion of human pathogens. It was shown that Caco-2 and HT-29 cultures are suitable models for determination bacteria adherence. The mechanisms of adhesion and the role of environmental factors in bacteria attachment are discussed. The comparative studies on the bacteria adhesion to epithelial cells and solid substrates are described. The cell lines are also used in the studies on the trans-epithelial transport of different food compounds. In the review the results of experiments on the food compounds bioavailability are presented. Enterocyte-like cells from the *in vitro* cultures are also used to study carcinogenesis process caused by some chemical substances and free radicals from food products. The biological effects of the DNA damage on intestinal cell necrosis and apoptosis are discussed. Further research should confirm that epithelial cell cultures cultivated in laboratory conditions have qualities similar to those present under natural conditions in the human alimentary tract.

INTRODUCTION

Food is a source of components which are indispensable for bodybuilding, reconstruction of human tissues and energy which, in turn, is necessary to maintain the living functions of the organism. Recently, much attention has been paid to the regulatory role of nutrients in metabolism, disease initiation and development. As a result, a new term – functional food – has been coined. This includes those components whose consumption may directly affect human health. Among those components there are some peptides, natural antioxidants as well as pre- and probiotics. A number of research have been devoted to these substances – they are the focus of contemporary science of human nutrition.

Among the research into functional food, an important role is played by these concerning the bioavailability of active agents, which is understood as preserving the biological activity of the substances in the alimentary tract and their absorption through the intestine walls into the blood circulation system. This research is very difficult to handle, as it is impossible to observe the inside of intestines, and taking samples for analysis is very complicated. Consequently, there is a need to develop models for *in vitro* research which would copy the conditions of the alimentary tract. Continuous advances are being recorded in this field – it is now possible to employ *in vitro* simulations which make the conditions of an experiment close to reality.

Among the major achievements is the development of an artificial alimentary tract, constructed by Dutch scientists (TNO – The Netherlands Organization for Applied Scientific Research, TNO), which perfectly imitates the process of digesting food. The apparatus is able to imitate the secretion of gastric and intestinal juices, dosing of secretes of the glands of the alimentary tracts (pancreatic juice, bile), absorption of digested food through the intestinal walls and peristaltic movements of intestines. Simplified versions of the "artificial" alimentary tract are used, which include incubation of food in solutions of enzymes and bile salts; the system sometimes ends with fermentors containing intestinal microflora.

Another field of research, which is the main subject of this literature review, are models of tissue cultures of intestinal epithelium, used in testing bacterial adhesion and in assay of selected biological activities as well as the absorption of various chemical substances, mainly food and medications.

STRUCTURE AND FUNCTIONS OF INTESTINAL EPITHELIUM *IN VIVO*

Besides the stomach, intestine plays a major role in digesting and absorbing food. Intestine walls have a laminar structure. On the inner side of the intestines there is a thin, strongly corrugated single layer of epithelium cells, called enterocytes; they cover the so-called "intestinal villi".

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Besides connective, muscular and nervous tissues, epithelium is one of the four main types of tissues found in a human organism. Their characteristic feature is a polar structure of cells, with separate parts – basolateral (deep) and apical (from the inner side of intestines). Their shape is elongated and cylindrical, with the nucleus placed in the bottom part. The upper part of the cell membrane, from the inner side of intestines, creates the so-called "brush border", which consists of numerous outlets – 1 μm-long microvilli. Creating the border is the proof of the correct polarisation of cells and of differentiating cells with a view to creating epithelium. The observation of alkaline phosphatase activity is a commonly used marker of the brush border-creation processes. Enterocytes are surrounded by a thick layer of glycocalix (glycoproteins and polysaccharides). Thanks to the brush border, the area of the intestine is increased 20–30 times [Cichocki *et al.*, 1996]. In the intestine of mammals, a single enterocyte can create a brush border in the apical part, covered by numerous microvilli, whose number can reach 3,000. This means that in each square millimetre there are up to 200 million microvilli [Jasiński & Kilarski, 1984]. From the side of microvilli, intestine walls are in contact with nutrients and this is where ions and organic substances are absorbed and certain substances are secreted to the intestine insides.

Enterocytes secrete large quantities of enzymes, a large portion of which cumulates in glycocalix. These include dipeptidases, alkaline phosphatase, oligo-1.6-glucosidase, glucoamylase, saccharase, lactase, maltase, enterokinase and intestinal lipase. The activity of these enzymes increases when chyme enters the intestines. The enzymes cause further decomposition of absorbed food and prepare it for transport to the deeper layers of intestine walls. Monosaccharides and amino acids are introduced to enterocytes by means of active transport and, after being modified, are transferred to capillaries in a basement lamina. Fatty acids and glycerol infiltrate into cells and are transformed into triglycerides within the smooth reticulum. After that, they bind with proteins in the Golgi apparatus. The resulting lipoproteins, making small droplets (chylomicrones), are secreted to the subepithelial layer and transferred to the lymphatic vessels.

Epithelial cells contain medium-sized filaments made of keratin. Their production depends on the type of epithelium, cell differentiation and the speed of their growth.

Epithelial cells are densely fixed on the basement lamina, creating a continuous, dense membrane, containing intercellular connections – desmosomes. One of the basic functions of epithelium is protecting vascularized tissues against mechanical damage; hence the ability of intestinal epithelium to regenerate quickly. Enterocytes are formed in crypts among villi. Enterocytes created therein migrate towards the villi, maturing and differentiating along the way. Epithelium can be regenerated after as little as 20 minutes; however, average lifetime of enterocytes is 2–5 days.

Sparsely spaced among epithelial cells are single goblet--like cells, acting as mucous glands. They are cylindrical in shape and widen in their upper part, becoming similar to mace. In their widened part, there are grains of mucus which consist mainly of glycoproteides (mucin) and are secreted outside. After absorbing a large amount , it creates a hydrophilic membrane on the intestine surface. Many proteins which take part in digesting and transporting nutrients into the organism are accumulated in mucus. Intestinal mucus protects enterocytes from being digested by intestinal juices. It is also a layer to which cells of intestinal bacteria adhere, creating a characteristic bacterial membrane. Moving towards the large intestine, the number of calicula increases significantly. In the large intestine itself, the ratio of enterocytes to goblet cells is 2:1.

The outer surface of epithelium is where an intestine contacts antigens. Hence epithelium contains cells which take part in immunological reactions. They form large clusters of gut-associated lymphoid tissue (GALT). In intestines, they form Peyer's patches, gathered in the subepithelial layer, but through the base lamina they get to the surface layers, ending with M cell (microfolds). They are lower than cylindrical cells and do not have microvilli, only folds. Their large portions are situated in the ileum. They are able to absorb large antigenic cells by endocytose and present them to the cells of the immunological system. Under the M cells there are lymphocytes B and T.

Below, under the layer of enterocytes, there is a base lamina. It is separated from enterocytes by a thin layer of mucopolysaccharides (glycocalix). The structure of the base lamina is not uniform. It is made of three layers: (1) inner loose lamina, (2) dense lamina, and (3) outer loose lamina. The lamina is composed of saccharides and proteins. The dense layer is made up of collagen type IV, laminin and proteoglycans. Laminin plays an important role of binding enterocytes to the base lamina. It is characterized by high affinity to receptors on the base side of enterocytes, to collagen type IV and to proteoglycans. The lamina is usually produced by enterocyte cells and forms a base layer on them, thus enabling permeation of nutrients and oxygen. Blood vessels (veins and arteries) and lymphatic vessels are situated in this layer. Under the mucosa there are two layers of muscles, making the intestinal walls more rigid and stimulating peristaltic movements. The first of them is made up of orbicular muscles, the other – of longitudinal muscles.

Epithelium cells create the main barrier separating chyme from a human organism. However, their function is much more complex. The directed transport takes place through the intestinal epithelium. It includes water and ions homeostasis, absorbing nutrients from intestines and secreting metabolites. Also, the epithelium surface is a place of adhesion of intestinal bacteria; it also prevents pathogenic bacteria and viruses from entering.

The small intestine is able to secrete large amounts of juices, reaching a volume of up to 6–8 L in 24 h. Much of the water secreted by the intestines (*ca*. 80–90%) is absorbed back. Only about 2 L of fluids enter the large intestine, where they are further absorbed together with mineral compounds. At the same time enterocytes can secrete large amounts of fluids abundant in Cl^- ions, thus cleaning the intestine surface.

CELL CULTURES OF INTESTINAL EPITHELIUM *IN VITRO*

Methods of culturing epithelial tissues were not developed until mid-1970s and the main developments in this field of research took place in the 1980–1990s. Initially, two approaches were suggested: (1) *in vitro* culturing of normal

enterocytes [Quaroni *et al.*, 1979] and (2) differentiating of malignant tumor cells of the large intestine into cells similar to enterocytes, induced by known factors causing the morphological and functional differentiating of normal and neoplastic cells, such as sodium butyrate, galactose or dimethylsulfoxide [Dexter, 1977; Dexter & Hager, 1980; Dzierżewicz *et al.*, 2002; Kim *et al.*, 1982; Pinto *et al.*, 1983]. The second approach proved particularly effective, which resulted in obtaining the established enterocyte-like lines. Using the Caco-2 line as an example, Pinto *et al.* [1983] proved that neoplastic cells can be differentiated spontaneously at a late stage of confluence. It was also observed that the lines transform into some forms resembling enterocytes with/in the developed brush border and produce enzymes appearing in normal enterocytes at the stage of border creation, like alkaline phosphatase, saccharase and aminopeptidase. Under natural conditions, the enzymes are the most active in the small intestine and less active in the large intestine. Their activity in *in vitro* cultures does not increase until the final stages on enterocyte culture; however, for phosphatase and saccharose it is lower by 50–70% than in intestines, and for aminopeptidase it is lower by as much as 90%. Alkaline phosphatase is produced by the apical cell membrane [Hidalgo *et al.*, 1989]. Bolte *et al.* [1998] determined the activity of phosphatase in membranes of the Caco-2 brush border and found it to be 192.6+/-79.8 mU/mg protein, whereas the activity of enzyme in membranes *in vivo* was 1592.8+/-704.6 mU/mg protein.

In the cell lines used, *e.g.* Caco-2, quick proliferation follows a short, 48-h period of rest and after 6 days a total confluence of the surface of the culturing bottle; after 9 days the culture enters the stationary phase of growth. With sufficiently frequent medium replacement, an enterocyte culture can be maintained for up to a month. At the end of the culturing period, cell's monolayer detaches from the medium, mainly at the bottle edges.

With the culture growth, microvilli are created. At the first stage of border creation, a monolayer of polarized cells is created with intercellular connections established between them. Meanwhile, the height of cylindrical cells gradually increases so much that in a 19-day-old culture they are 3.5 times as high as during the first two days of growth. As a competition to this process, clusters of cells are formed, which are covered by microvilli. They are filled with filaments built of a protein called "actin" [Peterson & Mooseker, 1993]. In the initial stage, about 70% of cells are covered with villi; in the final stage the whole culture in its apical part is covered with brush border. Microvilli come in two morphological forms: in half the cells the villi are distributed evenly, forming a carpet, whereas in the other half groups of villi form structures similar to flowers, in which particular villi seem to be connected in their upper part [Pinto *et al.*, 1983]. During the confluence, dome-like clusters of cells are formed, which after the first day of culturing reach the density of over 100 units in each square centimeter, and at the later stages their number is reduced by half. Dome-like concentrations are not found in cultures HT-29. Cells of this line do not create characteristic villi. It is supposed that the exceptional ability of the Caco-2 line to form a brush border may be associated with the similarity of neoplastic cells to embryonic ones. Both these types of cells contain glycogen in high concentrations, which is not found in other cell lines.

Developing methods of *in vitro* cultures has brought numerous advantages: it has become possible to conduct more detailed research into cell metabolism under more controlled conditions, research with the use of human tissues has become possible and it is now much more ethical than the research conducted on experimental animals [Shaw, 1996]. It must be stressed that there are serious drawbacks associated with the research with *in vitro* cultures. With each passage, the cultured cells are more and more different than the initial phenotype. Significant differences in the morphology and biochemistry of these cultures become visible. In such cases, two other types of *in vitro* cultures are suggested: organ cultures or histotypical/organtypical cultures.

Cell lines are derived from explants of natural tissues, taken from a living organism. Intestinal explants are transferred to special media with antibiotics, where they become "unbound". This is done either mechanically or by gentle homogenization or by hydrolysis with trypsin. After separating single cells from the remnants of the tissue, the so-called "primary" culture is obtained, which has the form of a cell layer adhering to the walls of the culturing vessel.

The primary culture transforms into diploid culture after 2–3 passages on fresh medium. The best material to derive such lines is embryonic tissue, although it is also possible to derive them from adult cells. A diploid line is such in which 75% of cells have the identical karyotype as the cells of the species from which they originate. Such cells have a double number of chromosomes. This form of cells is regarded as normal. In the consecutive passages, the diploid line steadily grows. In the first passages, the diploid cells preserve their primary epithelial morphology; however, after further passages they are significantly changed. Sometimes diploid cells become aneuploidal. They are characterized by a loss of sensitivity to stimuli associated with normal growth. They lose the ability to adhere to the surface and their growth is hindered by large densities of cells.

Usually after 50 generations (Hayflick's number) diploid cells die or undergo a neoplastic transformation and turn into a continuous culture. A characteristic feature of the established lines is their ability to reproduce in the consecutive passages. An established cell line is one which has been passed over 70 times. Continuous lines may be obtained directly from neoplastic cells or derived *via* diploid lines from normal tissues. The established lines grow very well, they usually have very low requirements and are therefore frequently used in biotechnology. The most commonly known lines of human intestinal epithelium are Caco-2 and HT-29.

It should be stressed that all the cell lines derived from adenocarcinoma and other neoplastic tissues are included in class II in terms of biological safety; therefore certain procedures must be observed in their culturing and storing, as well as properly-equipped laboratories must be provided, *e.g.* having laminar cabinets class II.

MAIN CELL LINES

Caco-2

Caco-2 is the most commonly used and the best described cell line (Table 1). It is stored, among others, at

the American collection ATCC under the catalogue number HTB-37 and at the German collection (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig) under the numbers: ACC 169, ICLC HTL97023, BS TCL87, 86010202, and HTB-37. The line was isolated from a neoplastic tumor of the large intestine of a 72-year-old man of the Caucasian race. It is an established line of adenocarcinoma. It is able to grow adherently on solid surfaces and on microporous membranes. During the culturing, it differentiates spontaneously. After 2–3 weeks, with full confluence, it makes up a monolayer of highly polarized cells whose structure is typical of enterocytes, with the nucleus situated in the base part, with dense mitochondria and the brush border in the apical part. Due to this type of structure, it is used as a model enterocyte line in *in vitro* cultures. Caco-2 cells produce enzymes (disaccharidases and peptidases) and transport proteins, typical of absorbing cells of epithelium. They are resistant to HIV and LAV viruses.

A part of the cells is polyploid. Caco-2 cells are able to express genes for protein I which binds retinoic acid and protein II which, in turn, binds retinol. They also have a receptor for thermostable enterotoxin (*Sta*, *E. coli*) and epidermal growth factor (EGF).

HT-29

In the collections of tissue cultures there is also the HT-29 cell line (*e.g.* ATCC: HBT-38; DSMZ: ACC 299). The HT-29 line comes from a 44-year-old woman of the Caucasian race and, like Caco-2, was isolated from a large intestine adenocarcinoma tissue. In *in vitro* cultures its morphology is typical of epithelial cells, but it does not differentiate to brush border-creating forms. A large portion of the HT-29 cell line population is made up of – goblet-like cells; hence this line produces large amounts of mucin. Its ultrastructure contains microvilli, microfilaments, strongly vacuolated mitochondria containing dark granules, endoplasmatic reticulum with free ribosomes, drops of lipids and a large number of lyzozymes. HT-29 cells produce a receptor for urokinase and vitamin D, but they do not have a receptor for plasminogen activator. There are numerous oncogens in the genome, *e.g.* myc+, ras+, myb+, fos+, and p53+. Mutation G → A was found in gene p53, which resulted in replacing arginine with histamine. Over-production of antigen p53 was also observed. In line HT-29, there are numerous isoenzymes: Me-2,1; PGM3, 1–2; PGM1, 1–2; ES-D, 1; AK-1,1; GLO-1, 1–2 and G6PD, B (catalogue ATCC, the Internet). Line HT-29 is sensitive to HIV and LAV viruses.

CULTURING VESSELS

Epithelial cells are typical adhering cells. This means that they start growing after being fixed to a solid surface and the growth continues until the total confluence of the whole culture surface.

Plastic T-bottles and multi-well plates for microtitration are the most frequently used in epithelial cell culture. In order to increase culturing surface, the surface of T-bottles may be corrugated. Most culturing vessels are made of polycarbonates.

Research into transporting of chemical substances through the epithelial layer uses cultures grown on membrane filters (0.1–12 μm), ensuring appropriate porosity and permeability for media and cell metabolites. In this type of culturing, Transwell (Costar), Falcon (Becton-Dickson) and Millicell (Millipore) type filters are used. Culture filters are made of polycarbonates, polystyrene, polyethylene tereftalate (PETF), and cellulose derivatives. Polycarbonate membranes are particularly recommended.

Culture substrate has a great effect on cells morphology [Shaw, 1996]. When growing on a solid surface (*e.g.* T-bottle), Caco-2 cells tend to detach and their polarization is difficult. When cultured on permeable membranes, they grow thickly, yielding pillar-like cells, which are typical of natural tissue. They are easily polarized and enable the tissue to differentiate correctly. The type of surface of the carrier on which enterocytes are fixed largely affects differentiation of cells. It is very advantageous if the culturing surface is covered with substances imitating natural base lamina. The most frequently used are collagen membranes (type I/III or IV), laminin or natural extracellular matrix (*e.g.* Matrigel). Artificial substances may also be used, *e.g.* collagen gels or collagen-glucusoaminogycane membranes.

Professional companies (*e.g.* Corning) offer sophisticated culturing systems in which a culturing chamber is divid-

TABLE 1. Characterization of human intestinal epithelial cell lines.

Cell line	Origin	Morphology	Secretion	Application	Collection
Caco-2	Human colorectal adenocarcinoma isolated from colon cancer of 72-year old Caucasian man. Absorptive cells.	Epithelial cell, adherent growth, forming brush border.	Enzymes: amino-peptidase, saccharase and alkaline phosphatase.	Study on bacterial adhesion, transmembrane transport, bearing down pathogenic bacteria by probiotic bacteria, protection against invasion of pathogens.	ATCC (USA), DSMZ (Germany), LGC (U.K.)
HT-29	Human colorectal adenocarcinoma, isolated from colon cancer of 44 year old women. Goblet cells.	Epithelial cells, adherent growth, not forming brush border. More permeable than Caco-2.	Secretion component of IgA, carcinoma--embryonic antigen (CEA), mucin.	Study on bacteria adhesion.	ATCC (USA), DSMZ (Germany)
T84	Cells of intestinal crypt	Epithelial cells, adherent growth, 2–3 layers, forming brush border.	Enzymes: alkaline phosphatase and saccharase.	Study on adhesion of food proteins.	ATCC (USA)

ed with microfiltrating membrane; thus a constant exchange of medium or gases is possible on both of its sides. The culturing chamber is thermostatic and equipped with a pH electrode and one for measuring the electric conductivity of a medium.

MEDIUM

The chemical composition of a medium for culturing epithelium cells is very complex. Its role is to ensure the correct nutrition of cells, metabolism regulation and cell differentiation. To this end, they must copy the composition of the systemic fluids, which are their growth environment. Two basic types of media are used: with serum and without serum. Because the cost of components is high, only those which are indispensable for tissue development are used. Serum is among the most expensive components. It has been found that both cell lines, Caco-2 and HT-29, are dependent on serum. It is a source of the necessary growth and regulatory factors for mammal cells. Natural components are expensive, but their use may also result in infections with mycoplasms and viruses. For the past ten years, there has been a clear tendency to replace natural media with chemically-modified ones. They are generally known as serum-free media.

All media contain carbohydrates, amino acids, vitamins, hormones, mineral salts, and growth factors. Glucose is the basic carbohydrate. It is an energy-providing component. Another one is glutamine which is added in the concentration of 2–4 mmol/L. Both energetic components are quickly consumed by cells and usually limit the growth of the cell population.

Mineral salts, apart from being nutrients, also regulate the osmotic pressure of liquids. It should be isotonic, which corresponds to about 300 mOsm/kg. Fluctuations of osmotic pressure should not exceed 10%. Sodium bicarbonate, together with 5–10% CO_2, is used as a buffering agent, keeping the pH value from 6.9 to 7.4. The Hepes buffer (25 mmol/L, pK 7.0) is also used for adjusting the pH value. In such cases, the concentration of CO_2 can be reduced to 2%.

Vitamins and hormones act primarily as cofactors. Their concentrations are different, depending on the cultured cell line. In Eagle's medium, commonly used in culturing human enterocytes, an important role is played by glutamine. It is the main oxidant and its use by cells results in a 5-fold increase in the production of carbon dioxide as compared to the use of glucose. It takes part in the production of purines and pyrimidines. Glutamine is preferentially taken up by quickly-growing cells. It is an obligatory component in serum-free media; there is a quantitative relationship between the concentration of this amino acid and the rate of cell growth in line Caco-2 ($r=0.87$, $p>0.01$). However, if serum is present, glutamine can be left out [Wiren *et al.*, 1998]. For the HT-29 line, the correlation between glutamine and the growth is significant ($r=0.68$, $p<0.05$) only in media with fetal bovine serum and in the absence of glucose. This means that glutamine does not affect all cell lines in the same manner.

Sera, which are obtained by centrifuging cells from blood, are added in up to 10% v/v. They are a rich source of nutrients and regulatory substances. Serum is a very complex substance. One of the best is foetal calf serum which contains an embryonic growth factor. Considering the high cost of this substance, attempts are made at reducing the amount of the serum added. One of the postulates is the reduction of the amount of the foetal calf serum to 2% by enriching it with iron and such additives as human transferrin, bovine insulin, dexamethasone, EGF, ascorbic acid, cholera toxin, and bovine mucus extract [Lentz *et al.*, 2000].

In vitro cultures are protected from a microbiological infection by an addition of a complex of antibiotics (penicillin, streptomycin).

The most frequently used medium for the Caco-2 cell line is the minimal Eagle's medium (MEM) enriched with Earle's solution: 2 mmol/L L-glutamine and 1.5 g/L sodium bicarbonate, 0.1 mmol/L non-exogenous amino acids and 1.0 mmol/L sodium pyruvate (total of 80%) and foetal bovine serum (20%). The use of MEM modified by Dulbecco is also recommended. The content of amino acids and vitamins is increased four times, whereas the concentration of glucose is 4.5 g/L. The medium is supplemented with 10% foetal bovine serum, 0.1 mg/mL streptomycin, 100 units of penicillin, 10 mmol/L HEPES buffer as well as non-exogenous amino acids and L-glutamine.

McCoy's medium 5A (90%) with foetal bovine serum (10%) is recommended for culturing HT-29 cells.

Attempts have been made recently at applying serum--free media [Wiren *et al.*, 1998]. One of such sera, commercially available, is Ultroser G, IBF. Usually, serum is replaced with a mixture of hydrocortisone, transferrin, insulin, ethanolamine, selenite and other active components.

Substances which affect the regulation of proliferation and differentiation of epithelium are added to the media for epithelium culturing. They include hydrocortisone (endocryogenic hormone), calcium ions, some vitamins (*e.g.* retinoic acid), growth factors (TGF-β), and others. These substances are added to a medium directly or obtained from culturing other cell lines (medium after astrocyte culture), or enterocytes are cultured together with other cells (*e.g.* fibroblasts).

METHODS OF CULTURE

Enterocytes are cultured in incubators with an adjustable level of carbon dioxide, at 37°C and pH 7.4, in the atmosphere containing 10% CO_2 with relative humidity exceeding 95%. A 30 mL portion of the medium is introduced to 75 mL T-bottles and inoculated with enterocytes in the amount of $10^5/cm^2$ per bottle. Caco-2 cells adhere strongly to plastic surfaces and grow quickly. Confluence of 75 mL bottles lasts 2–3 days. Every second day the medium is replaced with a fresh portion.

When 96-well plates are used, it is recommended that 75 μL of cell suspension with a density of 160,000 cells/mL are applied to a hole when a 21-day-old culture is used, and with a density of 460,000 cells/mL when a culture is 10 days old.

The coherence of a cell monolayer may be evaluated (1) visually under an inverted microscope, by observing the bottom of a T-bottle covered with cells or with a fluorescence microscope with fluorescence markers for dyeing nuclei, cell connecting protein ZO-1 (streptavidin-Texas red) or actin (rodamine); (2) by measuring the increase of trans-

membrane electric resistance of the medium with a special electrode (Millicell ERS Millipore or EVOM with WPI); and (3) by measuring the transport speed of ^{14}C or ^{3}H-labelled mannitol through a layer of enterocytes.

After about 80% confluence has been achieved, cells are detached by means of trypsinization. It is superfluous to strive to achieve 100% covering of the surface because with a lack of free surface cell growth slows down. The trypsinization procedure is carried out with a mixture of 0.03–0.2% EDTA at pH 6.4 with 0.25% trypsin in phosphate buffer without calcium and magnesium ions. Trypsin action lasts for about 5–10 min and is interrupted by the addition of a fresh portion of the medium. One 75 mL T-bottle yields *ca.* $1–2\times10^{7}$ cells.

A suspension of detached cells is used as an inoculating agent for maintaining the culture. Cells are centrifuged at 500 g for 5 min and suspended again in a fresh medium. However, it should be borne in mind that despite the carcinogenic origin of the Caco-2 line, in the consecutive suspensions it gradually degenerates, hence the culture should not be passaged more than 50 times.

To maintain the culture in a good condition it is necessary to set up a bank of cells by freezing a large number of samples with the youngest possible Caco-2 culture in liquid nitrogen. A practical rule should be observed: after obtaining cells (*e.g.* purchase from ATCC) the first large culture should be frozen. Small ampoules-cryotubes (1–2 mL) with a cap are used to this end. After centrifuging, enterocyte cells are suspended in the solution: 40–70% medium, 20–50% bovine or calf fetal serum and 10% DMSO or glycerol [Shaw, 1996]. Usually, $1–2\times10^{6}$ cells/cm^{2} are introduced to one ampoule. After being closed, the ampoules should be immediately put in a special polystyrene box and placed for at least 12 h at a temperature of -70°C. The purpose of this operation is to slowly freeze the suspension so as to avoid damaging the cells. Cryotubes are then placed in a Dewar flask in liquid nitrogen. Under such conditions they can be stored for many years. It should be noted, however, that cells frozen at -70°C can be stored for up to a month.

To defreeze the cryotubes, they should be placed in a water bath at +37°C and, after the ice has melted, the contents of the cryotube should be transferred to sterile centrifuge flasks with 10 mL of a fresh medium. The purpose of this action is the removal of toxic DMSO. After centrifuging, cells are again suspended in a fresh medium and a T-bottle is inoculated. If the correct procedure is observed, the full confluence should be achieved after 2–3 days. Throughout the entire procedure, a fixed rule should be observed: "quick freezing – slow defrosting".

Culturing HT-29 cells is similar to that of Caco-2. A new T-bottle is inoculated with 1:3 to 1:8 v/v of a mature culture after 3–6 days of growth. One T-bottle yields an average of $0.1–0.2\times10^{5}$ cells/cm^{2}. The number of cells doubles within 40–60 h. During the culturing, the medium should be replaced with a fresh portion every 2–3 days. Unlike Caco-2, the HT-29 culture grows more intensively and creates a well-developed brush border more quickly. Also, the HT-29 culture produces mucin mucus, which is a proof of the presence of goblet cells in the population of enterocytes.

Cultures should be frozen in liquid nitrogen in a solution made of fresh MEM medium (95%) and DMSO (5%) or a mixture of Coy's medium (70%), bovine fetal serum (20%), DMSO (10%), yielding *ca.* 3×10^{6} cells per cryotube.

APPLICATION OF *IN VITRO* CULTURES OF INTESTINAL EPITHELIUM IN RESEARCH INTO FUNCTIONAL FOOD

Research into adhesive properties of intestinal bacteria

One of the basic properties of intestinal bacteria is their ability to colonise the surface of intestinal walls. In newborn babies, the intestinal walls are sterile but they are quickly colonised by bacteria entering the body with food and from

TABLE 2. Cell viability tests.

Test	Determination method	Principe of determination	References
Trypan blue test	Microscopic observations	Blue dying of cells with damaged cytoplasm membranes.	
MTT (3-(4,5-dimethylthiazol-2--yl)-2,5-diphenyl tetrazolium bromide) reduction assay	Spectrofotometric assay	Reduction of yellow terazolium to blue dyed crystals of formazan only in mitochondria of living cells. Afterwards cell lysis, dissolution of formazon with detergent and determination of absorbance at 570 nm using microtitration plate reader (ELISA).	ATCC: MTT cell proliferation assay [Arredondo *et al.*, 2000]
Test with ^{3}H-tymidine	Measurement of radioactivity	Measurement of radioactivity in the medium under membrane covered with enterocyte monolayer.	
Kit, Molecular Probes, Leiden, The Netherlands	Measurement of fluorescence (fluorescence microscopy)	Hydrolysis of fluorogenic substrate calcein AM (2 μM in PBS) with esterase. Measurement of green fluorescence. Applied for tissue and cell analysis.	[Pietzonka *et al.*, 2002]
Test with homodimer-1 ethidium	Observation in light microscope	Nucleus dyeing to red colour with EthD-1 (4 μM in PBS) in dead cells.	[Pietzonka *et al.*, 2002]
Test with ethidine bromide and acridine orange (1:1)	Observation in microscope in UV	Dyeing of living cells to green colour and death cells to orange colour.	[Alwan *et al.*, 1998]

the environment. One of the first microorganisms colonising the surface of intestinal walls is *Escherichia coli*. These bacteria have an important ability to lower the redox potential in intestine; this creates favourable conditions for the development of other organisms. Among the bacteria colonising the surface of intestine in small children are bifidobacteria. Their development is favoured by acetylated saccharides in woman's milk [Newman, 1995] and transgalactosylated oligosaccharides in ready-made food offered by the food processing industry [Tannock, 1995]. At a later age, their amount stabilises, whereas in elderly people it decreases significantly [Mitsouka, 1984]. Ouwehand *et al.* [1999 c] proved that a decrease in the number of bifidobacteria in the intestines of elderly people results from weaker adhesion of bacteria to mucin. At an older age, the composition of mucin changes. It contains less protein and more carbohydrates, although the total amount of mucus secreted in people of different ages is alike.

In adults, the intestines are colonised by more than 400 species and the total number of intestinal bacteria reaches 10^{14} [Luckey & Floch, 1972]. This means that in an organism, the number of bacteria outnumbers the population of human cells by 10 times. Native microflora is predominated by gram-positive bacteria, anaerobes of the genus *Bacteroides*, *Eubacterium*, and *Bifidobacterium*. An important role is played by the genera: *Lactobacillus*, *Clostridia*, *Streptococcus*, *Fusobacterium* and *Peptostreptococcus*, which maintain the integrity of the mucin layer. They take part in the metabolism of mucin glycoproteins, bile acids, bilirubin, cholesterol, short-chain fatty acids, and produce nutrients for humans, *e.g.* menaquinons (vitamin K). Over 98% of the population of intestinal bacteria are absolute anaerobes, particularly those in the large intestine. Intestinal bacteria may be divided into native microflora, constantly present in intestines, and allochtonic microflora which reside there temporarily. The composition of intestinal microflora is variable. In particular sections of the intestine, a significant qualitative difference in species composition and in proportions between species may be observed. An important role is played by the age and nutrition of an individual. It was demonstrated in molecular biology that each person has a characteristic "bacterial profile". Numerous bacteria species have not been recognised because of the methodical difficulties in their isolation and imitating conditions of their growth.

Long-term studies have found that orally-administrated probiotics do not always colonise intestine permanently. The administered bacteria were very frequently found in faeces for several days; however, their number gradually decreased, which is a proof of temporary colonisation [Saxelin, 1997].

The main colonised surface of intestine walls is a mucus layer built of mucin. This substance is produced by goblet cells of the intestinal epithelium, which are particularly densely spaced in the large intestine. The intestine surface is very well developed thanks to intestinal villi, particularly so in the small intestine. Microvilli are covered with glycocalyx, which in turn is covered with mucin glycoproteins. Mucus layers on the epithelium surface create a liquid, dynamic coating, which is in constant movement.

Due to their immobilization, cells are more resistant to stress factors in intestines, and are more active metabolically. Numerous enzymes, secreted by a human organism to the alimentary tract, and taking part in digesting food, are adsorbed in the mucin layer, creating a favourable environment for the growth of bacteria. Bacteria cells find excellent conditions for contact with gut-associated lymphoid tissue, stimulating the production of antibodies, including anti-pathogenic immunoglobulins. The biological membrane, consisting of pro-biotic and neutral bacteria, creates an additional barrier that limits the contact of enterocytes with invasive microorganisms which can infiltrate the epithelium. On the surface of the mucus, probiotic bacteria drive pathogens out of their habitats, which is one of their functional features.

TABLE 3. Solid surfaces used to study adhesive properties of intestine microflora and pathogenic microorganisms.

Surface	Bacteria
Epithelial cell lines: Caco-2, HT-29, Intestine 407	*Bifidobacterium longum, B. animalis, Lactoacillus rhamnosus, Lb. paracasei ssp. paracasei, Lb. plantarum, Lb. acidophilus, Lactococcus lactis*
Isolated living mice enterocytes	*Salmonella typhimurium*
Mucin	*Lactobacillus rhamnosus GG, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus casei, Lactobacillus cremoris, Lactobacillus johnsoni La1*
Fibronectin	*Bifidobacterium longum, B. animalis Lactobacillus rhamnosus, Lb. paracasei ssp. paracasei, Lb. plantarum, Lb. acidophilus, Lactococcus lactis ssp lactis, Lc. lactis ssp. cremoris*
Collagen IV	*Lactobacillus acidophilus*
Laminin	*Lactobacillus sp.*
Matrigel	*Lactobacillus plantarum, Lb. rhamnosusu, Lactococcus lactis*
Expanded polystyrene	*Lactobacillus helveticus, Lactococcus lactis*

A particularly important role in the adhesive properties of eukaryotic cells is played by surface proteins situated on the surface of epithelial cell membrane and in adjacent matrix. Such proteins include fibronectin and various forms of collagen. These proteins are target sites for adhesion of intestinal bacteria. Bacterial proteins distributed in layer S manifest the high affinity to collagen and fibronectin, but extracellular polysaccharides excreted by bacteria at the initial stage of their growth may hinder adhesion. Lorca *et al.* [2002] pointed out the high affinity of bacterial proteins in *Lactobacillus* to collagen and to immobilised fibronectin; however, cells do not bind with these proteins when they are in a solution. Organelles, such as flagella, pili, secretive complexes, and adhesive organelle play a crucial role in bacterial adhesion [Knight *et al.*, 2000, Markiewicz, 1993]. All these outlet structures contain adhesins (lectins), which make up complexes with receptors on the enterocytes' surface. These interactions are highly specific. Adhesins are usually proteins, less frequently containing polysaccharides or teichoic acids. Adhesins recognise carbohydrate moieties in glycoproteins or glycolipides on the epithelial surface. Usually, these are the moieties of mannose, galactose and

fructose. Bacterial receptors also include fibronectin, certain albumins and immunoglobulins found on the surface of the intestinal epithelium.

The *in vivo* research conducted so far has shown active interactions of epithelial cells with intestinal microflora [Neish, 2002]. These connections are manifested, for example, by the surface of the intestinal epithelium secreting small amounts of cationic peptides, which act as antibacterial agents and control the number and distribution of intestinal microorganisms. If invasive bacteria appear and attempt to infiltrate the inside of enterocytes, an inflammation process starts. Infected cells secrete chemokins specific against neutrofils and leukocytes, which direct those cells to the infected spot. One such chemokin is interleukin-8 (IL-8).

One of the most frequently examined probiotic bacteria found in the small and large intestine of the human alimentary tract is *Lactobacillus acidophilus*. The crucial role in its adhesion to the cells of intestinal epithelium is played by its hydrophobicity and surface charge, as well as specific components, such as carbohydrates and proteins.

Conducting research into adhesion of probiotic bacteria *in vivo* is difficult as there are limited possibilities of carrying out experiments on human organisms. The only possibility is biopsies from the alimentary tract, performed during routine colonoscopy, and isolating micro-flora from post-operation material. Still, both these methods are inconvenient and of little availability. Consequently, model systems have been worked out to study the relationship between nutrients and microorganisms on the one side and the intestine surface on the other. Among the most commonly used are *in vitro* cultures of epithelial cells of Caco-2 and Ht-29 lines. The ability of these cells to differentiate, and the high similarity to enterocytes, enabled the use of these cultures as the biological equivalent of natural intestinal epithelium. The fact that both the lines, and particularly HT-29, are able to produce mucin was also of significant importance. A special subculture of HT-29, HT-29 MTX, in the presence of methoterexate, differentiates to goblet cells and produces large amounts of mucin mucus [Lesuffleur *et al.*, 1990]. The composition of the mucus is similar to that of mucin produced *in vivo*; it was used in the research into bacterial adhesion [Kirjavainen *et al.*, 1998; Tuomala, 1999].

Investigations into the adhesion of intestinal bacteria to enterocyte cells and to mucin have resulted in the publication of numerous papers. Tuomola *et al.* [1999a] showed that significant differences exist in the adhesion of probiotic bacteria to Caco-2 and mucin. Exemplary adhesion is displayed by *Lactobacillus bulgaricus* ssp. *cremoris* [Kirjavainen *et al.*, 1998], though this is a species, which poorly colonises intestines; on the other hand, cells of *Lactobacillus* GG [Elo *et al.*, 1991], *L. johnsonii* LJ1 [Bernet *et al.*, 1994], *L. bulgaricus* [Bianchi-Salvadori, 1986], and *L. lactis* ssp. *cremoris* [Lehto & Salminen, 1997] strongly adhere to enterocytes. These differences may result from those connected with receptors situated on the surface of enterocytes and in the mucin layer. Good adhesion of some bacteria directly to enterocytes is an indication that the adhering bacteria produce adhesins with affinity to epithelial receptors. A similar phenomenon occurs between bacteria and mucin.

What is notable is the strong adhesion of *Lactobacillus* to epithelium. This was shown in an experiment in which colonised epithelium was subjected to shaking. It was found that after an hour, few bacterial cells fell off. In order to remove the bacteria it was necessary to rip off bits of epithelium. The process probably takes place in intestines when food passes along them towards the rectum. Thus, on the regenerated epithelium, a new surface appears, ready to be colonised by bacteria; this process may underlie changes in the composition of intestinal microflora. Prolonged contact of some bacterial species with the human organism may determine their probiotic properties.

Research conducted by Tuomola [1999 b] showed that *Lactobacillus* adhere to enterocytes better than enteropathogenic bacteria. For example, the surface of intestines was colonised only by 1.4% *Salmonella typhimurium* and 0.5% *S. enteritidis*. Using Caco-2 culture as a model, Forestier *et al.* [2001] showed that the bacteria *Lactobacillus casei* ssp. *rhamnosus* (strain Lcr35) restrict the adhesion of enteropathogenic *E. coli* (EPEC), enterotoxic *E. coli* (ETEC) and *Klebsiella pneumoniae*, both in the case of pre-incubation of *Lactobacillus*, co-incubation and introducing *Lactobacillus* after colonising the epithelium by pathogenic bacteria. The researchers also proved that the post-culture fluid of Ler5 inhibited the growth of nine strains of pathogenic bacteria. The results of the research indicate that the strain L35 can be used to inhibit the colonisation of intestines by various pathogenic bacteria.

TABLE 4. Methods used for determination of adhesion of probiotic bacteria to enterocytes *in vitro*.

Experimental model	Mode of adhesion assay	References
Enterocyte monolayer	Radiolabelled of bacteria obtained by the use of labelled nutrient, *e.g.* ^{3}H-adenine. Incubation of labelled bacteria with enterocyte monolayer. Washing out of free cells. Determination of radioactivity of monolayer/bacteria complex.	[Cohen & Laux, 1995]
Microtitration plates coated with enterocyte cells	ELISA assay with the use of monoclonal antibodies. Wells covered with lysine and glutaraldehyde and coated with enterocytes. Adhesion of bacteria and determination using specific antibodies.	[Alwan *et al.*, 1998]

Scientists are not unanimous about the optimal age of enterocyte cultures for research into bacterial adhesion. 10-, 21- and 30-day-old cultures are usually used. The number of passages is not standardised either. Some authors claim that studies with cultures of not more than 25 passages are the most advantageous, whereas others point to the 100th passage. Briske-Anderson *et al.* [1997] studied physiological and morphological changes in Caco-2 cultures between the 20th and 109th passage. Transepithelial electric resistance (TEK) and saccharase activity increased within 36 consecutive passages, but the value of TER started to decrease significantly after the 60th passage, while the activity of the enzyme was still high, though strongly variable. When comparing cultures of various passages it was found that there are differences in TER but only until 15 hours of culturing in each passage; later, its values are similar regardless of the passage number. It was also observed that cultures from older passages reached a plateau earlier.

The number of cells of probiotic bacteria binding to each Caco-2 cell usually ranges from 0.005 to 2, and is rarely larger. It should be stressed, however, that other cell lines may be different. For example, the number of cells of line Int-407 reaches 4–7 [Blum *et al.*, 1999]. This indicates a limited number of receptors on the surface of the enterocytes, which could interact with bacterial cells.

There are few papers on the effect of normal intestinal microflora on intestine colonisation by probiotic bacteria. Research conducted by Ouwehand *et al.* [1999d] indicates that intestinal microflora does not affect adhesion of probiotic bacteria *in vitro*. Nevertheless, the adhesion process has been proved competitive in mixed cultures.

The environment largely affects adhesion of bacteria to the cells of intestinal epithelium. Still, no standardised conditions of carrying out tests of bacterial adhesion to the cells of intestinal epithelium have been presented in scientific literature. Different authors use different cell lines, different media for culturing, various buffers and pH values and bacterial cells in different phases of their growth. This makes comparing results and drawing general conclusions difficult.

One of such factors is the presence of polyunsaturated fatty acids (PUFA) which are produced in the large intestine by many bacterial species or ingested with food. It is noteworthy that lipolytic enzymes may significantly increase the amount of fatty acids in some foods. Kankaanpää *et al.* [2001] used Caco-2 to study the adhesion of probiotic bacteria in the presence of fatty acids. Under normal conditions, the presence of polyunsaturated fatty acids inhibits adhesion, thus weakening the action of probiotic bacteria. In this research, *Lactobacillus* GG, *L. casei* Shirota and *L. bulgaricus* bacteria to be examined for their adhesion, were incubated with linoleinic, γ-linolic, arachidonic, α-linolenic and decosahexaenic acids at physiological concentrations. It was found that higher concentrations (10–40 μg PUFA mL^{-1}) inhibited the growth and adhesion of the tested bacteria to mucin, whereas the adhesion of *L. casei* Shirota was supported by the presence of γ-linolenic and arachidonic acids at 5 $\mu g \cdot mL^{-1}$. PUFA also changed the adhesive properties of Caco-2 cells, but this effect was multidirectional. With arachidonic acid, the adhesive properties of enterocytes deteriorated as compared to all the three bacteria. In *L. casei* Shirota, adhesion increased when α-linolenic acid was present. This data indicates that the presence of fatty acid significantly affects the adhesion of bacterial cells to Caco-2 cells.

In the research into transport of chemical compounds through intestinal epithelium, fragments of intestines taken from animals, mainly pigs, were used. Pietzonka *et al.* [2002] took samples of intestines directly from a slaughterhouse and transported them in ice blocks obtained after freezing a strongly oxygenated Krebs buffer (95 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 1.1 mmol/L KH_2PO_4, and 40 mmol/L glucose). Transport time did not exceed 45 min. In the laboratory, 4 sq. cm fragments were cut out of particular sections of intestines. Then they were gently washed with the buffer to clear off intestinal digesta, and placed in the holes of a culturing plate. The tissue was then incubated for 10 min in a buffer heated to 37°C; after that a proper study of trans-membrane transport was carried out. By employing a test with calcein AM – a fluorogenic substrate of esterase – hydrolysed to green fluorescing calcein (Molecular Probes, Leiden, Netherlands), the authors found out that the 80% vitality of cells was maintained only for 30 min; however, histological tests showed that after the explant had been defrosted, changes in cell structure took place immediately. This indicates that such tissues are useful for *in vitro* studies only to a limited extent.

Sarem-Damerdji *et al.* [1995] studied the adhesion of five species of *Lactobacillus*: *L. acidophilus*, *L. casei* GG, *L. plantarum*, *L. acidophilus* Ki, and *L. paracasei* subsp. ACA.DC212.3, to the surface of a piece of human intestine, obtained from post-operative material. The patient, who had had a piece of his intestine with adenocarcinoma cut out, had not been subjected to chemotherapy or antibiotic therapy. After pre-cleaning, the fragment was placed in a hosphate buffer, from which calcium and magnesium ions had been removed, and transported from the hospital to the laboratory at a temperature of 4°C. Subsequently, the mucin layer was cut off from the muscle layer and washed thoroughly with RPMI 1640 medium with antibiotics. Then, after placing on a porous membrane, the explant was cut with a scalpel into small pieces which were put into test tubes with 2 mL of medium. Following this, suspension of bacterial cells was introduced to the tubes and incubated for 8 h at 37°C. The free, non-adsorbed bacteria were then washed out and the cells strongly fixed to the intestinal tissue were counted. The authors proved that the bacteria *L. casei* GG and *L. acidophilus* NCFB 1748 did not adhere as strongly as other strains.

In recent years, there have been more and more papers devoted to research into the adhesion of probiotic and intestinal bacteria, using mucin (intestinal mucus). This may be obtained mechanically, by scrubbing it off gently from the intestine's surface; however, it is hard to obtain it from healthy individuals [Laux *et al.*, 1984]. Glycoproteins from the human small intestine are the main source of mucus, but it is not obtained from individuals typical of the healthy human population [Tuomola *et al.*, 1999 b].

Mucin can also be isolated from faeces [Miller & Hoskins, 1981]. To this end, faeces are diluted in a phosphate buffer at pH 7.2, with microflora activity inhibitors (NaN_3), serine proteases (phenylmethylsulphonyl fluoride), serine-containing enzymes (iodoacetamide) and metalproteases (EDTA). Then, after being cooled down to 4°C , the suspension is centrifuged for 30 min at 15,000 g; from the clear supernatant thus obtained, mucin is precipitated twice with ice-cold 60% ethanol. The obtained mucin is suspended in ultra-pure water and lyophilised [Ouwehand *et al.*, 1999b].

Mucin plays an essential role in adhesion of intestinal bacteria. Its physiological role consists, among others, in binding intestinal microflora and protecting intestinal epithelium from invasions of pathogens. It is supposed that mucin contains receptors resembling those of enterocytes, which causes it to be the first layer to which bacteria adhere. The layer of intestinal mucus is constantly renewed; it is produced by goblet cells. The mucus layer is also a barrier protecting intestine walls from infections by *e.g.* rotaviruses, which cause diarrhoea in small children.

In recent years, more and more papers have been dealing with adhesion of probiotic bacteria concerning intestinal mucus. One might say that in model research *in vitro*,

mucin drives out the system of enterocyte monolayer, which has been used to date. Kirjavainen *et al.* [1998] studied the adhesion of probiotic bacteria to the mucus isolated from the faeces taken from individuals of different ages (newborn babies, children and adults). The authors found out that the patients' age affects the effectiveness of mucin colonisation. Much stronger adhesion was observed in the case of mucus taken from adults than that taken from children. Depending on the mucus donor's age, the bacterial adhesion was: 44–46% for *Lactobacillus* GG, 23–30% for *Bifidobacterium* lactis Bb-12, 9–14% for *Lactobacillus johnsonii* LJ-1, 3–10% for *Lactobacillus salivarius* LM2-118, 2–3% for *L. crispatus*, and 3% for *L. paracasei* F19. The connection between adhesion of bacteria and the age of the individuals – mucus donors – was also pointed out by Ouwehand *et al.* [1999a]. In the case of *Bifidobacterium*, the effectiveness of adhesion of these bacteria to mucin decreases in elderly individuals and in very young ones.

Intestinal mucus was used by Juntunen *et al.* [2001] in research on adhesion of probiotic bacteria in diarrhoea. The mucin used in that research was isolated from 10-year--old children suffering from diarrhoea, whereas mucus obtained from healthy children, was used as a control sample. Adhesion was investigated for known probiotic strains, such as *Lactobacillus rhamnosus* GG, *L. casei* Shirota, *L. paracasei* F19, *L. acidophilus* LA5, and *Bifidobacterium lactis* Bb12. It was found that the adhesion of bacteria to mucin ranged from 1–34% and was similar in both cases: for the mucus taken from healthy children and from the ill ones; the highest adhesion was found for *L. rhamnosus* GG and *Bifidobacterium lactis*. Very good results were obtained for a mixed culture of these bacteria.

It should be stressed that all the models used are highly simplified and do not fully imitate the conditions existing inside intestines. Firstly, they are different from natural ones in terms of the composition of the media in the intestines, atmosphere of gases inside the intestines, the abundance of intestinal microflora, and mechanical conditions (peristaltic movements).

One of the most difficult problems is feeding the intestinal microflora. Most research conducted so far have been dealing with *Lactobacillus* sp., in culturing of which the MRS medium is commonly used. It is a very rich medium, but also partly selective. Its composition has nothing in common with the chemical composition of the intestinal digesta, particularly of the large intestine. In the case of the research into enterocytes, the conditions are even further from natural, as very rich media imitating body fluids, are used for research. Most researchers, who use epithelial models before introducing bacteria to enterocyte cultures, suspend them in media which are used in tissue cultures. It is surprising that in the literature there are practically no papers concerning the preparation of media imitating the contents of the intestines. This is one of the key issues in the research into intestinal microflora *in vitro*.

Another environmental factor, which differs from natural conditions, is the composition of intestinal gases. In the large intestine, there is practically no oxygen; gases are produced there, which are typical of methane fermentation. However, *in vitro* cultures of enterocytes must be oxygenated. The presence of oxygen has negative effects because many organisms colonising the intestines cannot be cultured in an atmosphere containing oxygen and in the rich media used so far for LAB and enterocytes (*e.g.* bifidobacteria).

Another element, which should be considered in the research into adhesion of microorganisms, is the effect of natural and rich intestinal microflora on the adhesive properties of single organisms used in the research. Peristaltic movements of intestines favouring the peeling off and regeneration of epithelium and movement of mucus with solid components of food – these are all difficult to imitate in a laboratory in rigid laboratory vessels.

The models used so far have resulted in differences in the results obtained during the research into bacterial adhesion *in vivo* and *in vitro*. It should be stressed that the models used are highly useful in research into adhesive properties of bacteria.

Binding nutritional components

In scientific literature there are very few reports on using the cultures of enterocytes *in vitro* to study the direct binding of nutritional components to intestinal epithelium. Bolte *et al.* [1998] examined binding of gliadin peptides, ovoalbumin and beta-lactoglobulin. In the tests measuring the binding of proteins, they used Caco-2 and T84 cell lines. Both lines came from the ATCC collection (USA). In these experiments, they used fractions of membranes with the brush border which were isolated from an enterocyte culture. The research included common incubation of membranes of the brush border with the above-mentioned proteins and determining the chemiluminescence of biotinylated proteins by the dot blot method. For ovoalbumin, the saturation of membranes took place at a concentration of protein reaching 50 μg/mL for both cell lines. Binding gliadin peptides and beta-lactoglobulin to the membranes of T84 brush border was saturated at lower concentrations (7 and 5 μg/mL, respectively) than in the case of the border in Caco-2 (50 and 10 μg/mL). It was shown that the affinity of nutrient proteins to the membranes of brush border of both enterocyte lines was of the following growing sequence: gliadin peptides – ovoalbumin – beta-lactoglobulin. The researchers concluded that owing to the similarity of Caco-2 and T84 lines to the cells of the brush border, they could be used as a model in studies into the adhesion of proteins to brush borders.

***In vitro* cultures as a model for studying invasions of pathogenic bacteria and viruses**

Adhesion is a critical stage in the development of most infections, including those of the alimentary tract walls. Numerous pathogenic bacteria show high invasiveness and penetrate epithelial cells, getting through the barriers of mucus and cytoplasmatic membrane and further into the deeper layers of intestine walls. Mechanisms of penetrating through the barriers of intestinal epithelium have been described for such animal pathogens as *Shigella* [Nhieu & Sansonetti, 1999], *Yiersinia enterocolitica* [Autenrieth & Firsching, 1996], *Listeria monocytogenes* [Daniels *et al.*, 2000], and *Salmonella typhimurium* [Clark *et al.*, 1994; Rice *et al.*, 1997]. All these pathogens manifest preferential ability to adhere to M cells, which are situated in Peyer's patches. Pathogens are accumulated in large amounts in Payer's patches and penetrate deep into tissues and to blood, and within 30 min they reach the internal organs.

The M cells cover the sacs of the lymphoid tissue surrounding the alimentary tract. They are found along the length of both the small and large intestines. Clusters of lymphoid sacs make up Peyer's patches. An attack by pathogenic bacteria is associated with the secretion of invasion proteins by the system of secretion type III. The proteins penetrate the enterocyte cells, rearranging their cyto-structure (protein Ipa A) and bringing about polymerisation of actin (proteins IpaB and IpaC).

An invasion of pathogens is hindered by the covering of intestinal epithelium by a layer of bacteria which restrict the access of pathogens to the intestine surface. This is one of the functional properties of probiotic bacteria.

Creating a bacterial barrier, which restricts access of pathogens to the epithelial surface, may be analysed with models of cell cultures *in vitro*. An interesting tissue system was proposed by Daniels *et al.* [2000]. They obtained M cells by induction of enterocytes Caco-2. To this end, they used a sub-clone of Caco-2, which created a clear brush border, similar to that in the small intestine *in vivo*. Enterocytes (3×10^5 cells) were grown on the surface of a filter (Transwell filters) until they had been fully differentiated (14 days). This was followed by introducing murine lymphocytes, isolated from Peyer's patches of Balb/c mice, on the lower part of the filter. In this way, a "sandwich" was obtained in which the upper part was made up of enterocytes and the lower part of lymphocytes. Both cell layers were separated by a 3-μm-pore filter. A co-culture was thus prepared was cultured for another 4–7 days, until enterocytes had been transformed into M cells (test FITC-conjugated latex bead, Moledcular Probes, Eugene, OR, USA). A pathogenic strain of bacteria *Listeria monocytogenes* was introduced onto the upper surface of modified enterocytes (M cells). *Yersinia enterocolitica*, known to adhere preferentially to M cells, was used as a reference strain. A microscopic analysis showed that L. monocytogenes rarely adheres to the surface of epithelial cells and its distribution among enterocytes and M cells is random, whereas the reference strain adhered mainly to M cells.

The infectious activity of bacteria *Listeria monocytogenes* was studied with the culture of enterocytes *in vitro*. While incubating a culture of Caco-2 with bacteria *L. monocytogenes*, Hauf *et al.* [1999] found an induction in enterocytes of the synthesis of three nucleoprotein complexes, similar to the NF-kB necrosis factor. Valenti *et al.* [1999] showed that an invasion of Caco-2 cells by these bacteria triggers changes leading to the necrosis of enterocytes. It was also shown that an addition of bovine lactoferrin reduces the extent of the bacterial invasion [Valenti *et al.*, 1999].

One of the invading pathogens is *Campylobacter jejuni*. It frequently causes diarrhoea. Its virulence is strictly associated with the ability to penetrate the cells of intestinal epithelium. Little is known about the mechanism of these bacteria penetrating eukaryotic cells. Wooldridge *et al.* [1996] studied the endocitosis of *C. jejuni* with a culture of Caco-2. They found that processing the monolayer of Caco-2 with filipin III, which chelates cholesterol in the cytoplasmatic membrane of enterocytes, strongly inhibits penetration of the epithelium by the bacteria. Among the factors inhibiting an invasion of *C. jejuni* they mentioned wortmannin, an inhibitor of phosphatidiloinositolic kinase, and cholera toxin.

A culture of Caco-2 enterocytes was also used to study the virulence of *Aeromonas hydrophila*. Guimaraes *et al.* [2002] showed that a non-enterogenic strain of *A. hydrophila*, isolated from the faeces of an 8-month child with diarrhoea symptoms, causes the vacuolarisation of enterocytes. It leads to damaging the monolayer and penetration of the intestinal tissue by the bacteria.

Numerous intestinal diseases are caused by a viral infection. A classic example is enteritis in children, caused by rotaviruses. On a global scale, over half the children hospitalised because of acute enteritis were infected by rotaviruses [Middleton *et al.*, 1977]. The Caco-2 can be successfully used for detecting the presence of rotaviruses. Cumino *et al.* [1998] used enterocytes to multiply rotaviruses. The cells were kept in PBS, suspended in Eagle's medium, enriched with bovine serum, and inoculated with viruses. The ELISA test, detecting viral proteins, was used to detect the presence of rotaviruses, along with electrophoresis on polyacrylamide gel. The method proved to be sensitive and reliable.

Study into bioavailability of nutrients and medicines

The main area where nutrients are absorbed in a human organism is the small intestine. It is *ca.* 2–6 m long, with duodenum constituting 5% of the total length, ileum – 50%, and jejunum – 45% of the total length. Food is transported along the small intestine within *ca.* 2 h with enzymatic digestion accompanied by intensive absorption. This takes place according to the mechanism of passive diffusion, facilitated diffusion, osmosis and active transport. Over 90% of nutrients are absorbed in the small intestine. In the initial sections of the intestine, particularly in the duodenum, passive absorption dominates, in farther ones, active transport proceeds. More detailed data on the anatomy and physiology of the small intestine have been compiled in publications by Stoll *et al.* [2000] and Ekmekcioglu [2002].

Under *in vivo* conditions, the exchange of mass between the intestinal digesta and the organism proceeds through intestinal epithelium. Due to this, epithelial cells are used as the main model in research into transepithelial transport *in vitro*. The model is used both by nutritionists and pharmacists to study the absorption of nutrients and medicines.

In research into transepithelial transport, cultures of enterocytes are usually used which grow on microporous membranes covered with a layer of hydrogel, *e.g.* collagen. Hidalgo *et al.* [1989] studied transepithelial electric resistance (TEER) of membranes covered with collagen and without collagen. They found that crosswise resistance of a polycarbonate membrane covered with collagen is 30–60 Ω, whereas for the same membrane without collagen – 5–10 Ω, which demonstrates the low resistance of the collagen layer. An increase in transmembrane resistance is a good marker of the differentiation of epithelial cells. Faulty Caco-2 and HT29 cells, with limited ability to differentiate, do not increase the resistance and are not suitable for research. Transmembrane resistance of a mature, well-differentiated culture of enterocytes is usually 180–250 Ω and is reached after 14–21 days of culturing. The transepithelial transport studies are usually performed using intestine cell monolayer with TEER value of 250–300 Ω cm^{-2}.

At normal confluence, the amount of chemical substances passing through a monolayer of enterocytes gradually decreases. Hidalgo *et al.* [1989] studied the transfer of

lucipherase and inulin. The researchers found out that a reduction in transfer of the compounds between day 1 and 10 was by 160 and 340 times, respectively. The data point to a progressive integration of the monolayer, which is a result of congestion and interrelations between enterocyte cells.

A crucial role in research into transmembrane transport is played by the full confluence of the membrane surface, which ensures its total integrity. This parameter has been explored thoroughly. Determination of the integrity of a monolayer consists in measuring the transfer of radioactively-marked substances, dyes or fluorescent compounds, which are usually not metabolised by cells (Table 5).

The very rule of testing is relatively simple. It consists in studying the transfer of a given substance through a monolayer of epithelial cells; the measurement involves determining the concentration of a given substance in the medium under the membrane, or indirect determinations, such as the measurements of: medium radioactivity, a level of fluorescence or the difference between electric resistance on both sides of the membrane.

Special multi-well plastic vessels with membrane filters of high porosity (usually 0.4 μm) are currently commercially available. The most commonly used vessels of this type are multi-well plates Transwell, manufactured by Costar, and Millicell manufactured by Millipore. Culturing vessels, the so-called "holes", are 12–24 mm in diameter, and in their case the volume of the medium over and under the membrane is only 0.5–3 mL.

Transport studies are carried out with special media. The first stage of culturing consists in the growth of enterocytes in DMEM medium. After the final stage of confluence has been achieved, the culturing medium is poured out and replaced with Hanks' balanced salt solution (HBSS) with HEPES buffer of pH 7.4, glucose and a complex of protective antibiotics. It should be noted that attempts have been made recently to develop a substitute for intestine fluids as a transport medium, which would replace HBSS, but without much success [Ingels *et al.*, 2002].

The characteristics of the diffusive properties of a monolayer of epithelial cells is determined based on the permeability factor [Artursson, 1990]:

$$P_w = V/AC_0 \, dC/dt \ [\text{cm sec}^{-1}] \qquad (1)$$

where: V – the volume of the medium on the side to which the component passes (cm^3), A – membrane surface area (cm^2), C_0 – initial concentration of the component on the side where it was introduced ($\mu g/cm^3$), dC/dt – concentration increase in time on the recipient side ($\mu g \ sec^{-1}$). The experiment is usually conducted for 1–6 h.

The results are given as mean values (±SEM) of at least six measurements. The differences in P_w between directions of diffusion or between various components are estimated based on Student's t-test at a significance level of $p<0.05$. In the research into the transport of a given substance, two-direction diffusions are examined – from the apical to the base and from the base to the apical side.

There are ample reports addressing the use of enterocytes *in vitro* in research into transmembrane transport. The research has one common feature – it is conducted only with model systems, where the transported compound is given in a chemically-pure form. While the use of models may be considered correct in research into the transport of medicines, in the case of nutrients it is a far-fetched simplification. It does not take into account the effect of the organic matrix of a food product either on the physiology of enterocytes or on the interactions of the matrix-component type. Another flaw of the models is neglecting the effect of

TABLE 5. Integrity tests for enterocyte monolayer examination.

Method	Labelling	Measurement	References
Test with horse-radish peroxidase	–	Peroxidase transport across enterocyte monolayer measured as enzyme activity under membrane	[Shaw, 1996]
Transepithelial electrical resistance (TEER)	–	Transepithelial electrical resistance measured with electrodes placed in medium over and under enterocyte cell monolayer. The value of this parameter reached 100–300 Ω for 10-day old cultures and 100–400 Ω for 21-days old cultures	[Briske-Anderson *et al.*, 1997; Hidalgo *et al.*, 1989; Millipore User Guide MultiScreen Caco-2]
Transfer of labelled mannitol	Radiolabelled ^{14}C-mannitol (m. w. 182)	Measurement of medium radioactivity under enterocyte monolayer with scintillation counter	[Briske-Anderson *et al.*, 1997; Lentz *et al.*, 2000]
Transfer of other radiolabelled substances	Radiolabelled ^{14}C-inulin, ^{14}C-poly-ethylene glycol (m.w. 4,000), ^{3}H-dextran (m. w. 70,000), ^{3}H-taurocholate ^{14}C-flavone	Measurement of medium radioactivity under enterocyte monolayer with scintillation counter	[Hidalgo *et al.*, 1989; Lentz *et al.*, 2000; Kuo, 1998]
Test of monolayer integrity with luciferin yellow	Luciferin yellow CH, (Sigma; m.w. 457)	Measurement of transepithelial transfer of luciferin yellow using ELISA fluorescence reader at λ_{EX} 450 nm, λ_{EM} 535 nm	[Hidalgo *et al.*, 1989; Millipore User Guide MultiScreen Caco-2]
Test with phenol red	–	Measurement of absorbance under enterocyte monolayer at 558 nm	[Briske-Anderson *et al.*, 1997]
Fluorescence test with propidine iodine	Induction at λ_{EX}=536 nm, emission at λ_{EX}=617 nm	Fluorescence of enterocytes observed in a microscope	[Shaw, 1996]
Labelled yeasts	Isothiocyanine of rodamine B	Fluorescence of cells in a microscope	[Pietzonka *et al.*, 2002]

intestinal microflora, which actively affects the entire intestine system *in vivo*. When the Caco-2 model is used, it should be borne in mind that a monolayer made up of these cells is much denser than is the case in natural intestinal epithelium. The research by Artursson *et al.* [1993] showed that transport through a Caco-2 layer is 1/20 of that through the large intestine and 1/100 of that through a human small intestine. So far, no clone of Caco-2 has been presented with higher transmembrane permeability. There are many reasons for the difference in permeability between a Caco-2 monolayer and a normal intestinal epithelium. Culturing *in vitro* is not controlled by the central nervous system, it is not normally supplied with blood, it is not properly covered with a thick layer of mucin and it is not vertically-corrugated in villi-crypts. The areas covered with goblet cells have a looser structure. This type of cells, apart from cylindrical enterocytes, is found in the HT-29 cell line. Unfortunately, there is no line consisting of goblet cells alone.

A population of clones of HT-29 line, cultured in different laboratories, may demonstrate different properties. For example, the HT-29H line consists of 80% of goblet cells capable of creating a mucin layer, which considerably impedes the transfer of lipophylic substances. This line also creates strong intercellular bonds, which results in its lower permeability compared to the Caco-2 line. Diffusive properties of the HT-29 line depend on the type of the medium used. Wikamn-Larhed & Artursson [1995] proved that the enterocytes from this line, which are cultured on RPMI medium, make up a looser structure than those cultured on DMEM. This may be the result of producing mucin during the growth on DMEM, whereas the mucin layer on RPMI is invisible [Wikman *et al.*, 1993]. The study conducted by Wikman-Larhed & Artussona [1995] on a co-culture of both lines proved that the obtained mixed culture displays permeability which is between the values for each of them separately.

Much of the research with enterocyte models address transport of antioxidants. One of the most important groups of such compounds are polyphenols which include a range of various chemical compounds.

Walgren *et al.* [1998] conducted research into the transport of quercetin through the intestinal epithelium using the Caco-2 enterocyte model. Quercetin is a glycoside commonly found in fruit and vegetables. It is a flavonol known for its anti-carcinogenic, antineoplastic, anti-sclerotic, and anti-clotting activity. In the alimentary tract, the quercetin glycoside, also referred to as rutin, is hydrolyzed by intestine microflora to quercetin and glucose. It is assumed that intestines adsorb only the quercetin aglycone, however, this phenomenon has not been fully elucidated so far. In their research, the authors used chemically pure quercetin, quercetin 4'-glycoside, and 3,4'-diglycoside. During a 60-min exposure, the transport of quercetin was found linear. The relative permeability for the epithelium was 5.8×10^{-6} cm·sec^{-1} and was over ten times as high as for mannitol. When the direction was reversed, from base to apex, the speed of quercetin transport doubled. Surprising results were obtained when 4'-glycoside was introduced at the apical side. In that case, no transport through the epithelium was observed. Although the incubation time was extended up to 3 h, no signs of the transport of this glycoside were observed, but its stability was very high and exceeded 95%. Unlike 4'-glycoside, quercetin 3,4'-glycoside is absorbed by epithelium on both the apical and base side; the speed of transport of this substance from the base side was almost five times higher. The results obtained by Walgren *et al.* [1998] were only partly corroborated by other scientists. Murota *et al.* [2000] also observed a rapid decrease in the concentration of quercetin on the apical side; however, their observations concerning glycosides are completely different. The transport of 4'-glycoside through epithelium was slow at first, but after 2-h incubation its concentration on the apical side decreased. The concentration of the other glycosides, *i.e.* 3.4'-di-glycoside and 3'-glycoside did not change. A better transfer of 4'-glycoside was likely to result from its higher lipophylicity. On the apical side, quercetin and isoramnetin conjugates accumulated gradually. This is a proof of metabolizing quercetin to conjugates and of their partial secretion in the apical direction. The conjugates of quercetin and isoramnetin were also observed to accumulate on the base side, with their total content accounting for 20% of the quercetin introduced on the apical side. Much of the isoflavones were accumulated in cells as aglycon and its conjugates. The inconsistent results obtained by various researchers show that it is necessary to continue research into the transport and metabolism of quercetin and its glycosides in the intestinal epithelium.

Caco-2 was also used to investigate the transepithelial transport of flavones [Kuo, 1998]. It is an important group of flavonoid hydrophobic antioxidants of plant origin, with anti-carcinogenic and anti-oxidant abilities. Epidemiological studies have shown that supplementing a diet with flavones reduces the incidence of the large intestine cancer. The paper by Kuo [1998] throws some light on the bioavailability of those substances when investigated with the use of a culture of human enterocytes Caco-2. Radioactive, ^{14}C- marked flavones at the concentration of 10 μmol/L (Proctor & Gamble Co., USA) were used in the research. Considering low solubility of flavonoids in water, they are usually used in concentrations not exceeding 100 μmol/L. An aromatic amino acid called "phenylalanine" was used as a reference substance. It was shown that the speed of transfer of flavones was five times higher than that of phenylalanine, and that the transport of flavones in both directions was similar. However, the transport of phenylalanine from the surface side of enterocytes to the base side was 10 times higher than in the opposite direction. It was also found that a considerable part of the flavones were accumulated in enterocytes. Their average concentration in cells was *ca.* 1 nmol/mg of proteins. Replacement of the buffer with a fresh portion resulted in transferring the accumulated flavones to the buffer under the membrane. Replacing sodium ions in the transporting buffer with potassium ions did not affect the speed of flavone transport. On the contrary, at lower incubation temperatures, introduction of potassium ions was found to considerably slow down the transport of flavones from the base side to the surface side. Introducing bovine albumin in the concentration of 100 μmol/L to the transporting buffer reduced the transport of flavones by half. This was a result of binding a large amount of water by the protein. The increase in flavone concentration from 10 μmol/L to 100 μmol/L did not affect the speed of their transport. At the concentration of 10 μmol/L, the transport of antioxidants from the base side to the apical side took *ca.*

90 min. The authors postulated that quick diffusion of flavones through a layer of intestinal epithelium may be the chief mechanism of their absorption into the human organism.

The Caco-2 model was also used to study the absorption of isoflavones found in soybean: genistein and daidzein and their glycosides. Steensma *et al.* [1999] introduced 50 μmol/L solutions of flavonoids to an enterocyte culture from the apical side. Considerable differences were found in the transport and metabolism of these compounds. After 6 h of incubation, 30–40% of genistein and daidzein were transferred to the base side and remained there for 24 h, without any quantitative changes. It was shown that permeation of the glycosides of these flavones through a layer of epithelial cells was difficult. Both glycosides were metabolized inside the cells to genistein and daidzein and about 20% of aglycons passed to the medium from the base side. This means that Caco-2 cells display endogenous glucosidase activity. A part of isoflavones was metabolized to the polar form, *i.e.* glucuronides and sulphates.

Reports have also been published on lesser-known flavonoids, such as chrysin, which is found in honey and propolis. Walle *et al.* [1999] conducted experiments on the transport of this antioxidant through a layer of Caco-2 cells and found that it diffuses from the apical surface towards the base surface at a speed of about 6.9 cm·sec^{-1}, whereas the speed of its diffusion in the reverse direction is twice as high. A high diffusion speed was observed only during the first hour of incubation, after that it had significantly dropped. Chrysine metabolites (glucuronide and chrysine sulphate) also diffused easily. Introduction of an anionic inhibitor hindered the transfer of those metabolites, which may suggest an easy mechanism of the transport of chrysine itself, but a more difficult adsorption of its metabolites produced by enzymes excreted by enterocytes.

Numerous studies, mainly in pharmacy, which used the Caco-2 model, have addressed the transport of peptides. When passing through the alimentary tract, they are hydrolyzed to amino acids; therefore, the bioavailability of whole peptides is estimated as <1%. The transport of peptides through a mucin layer to enterocytes is accelerated by bile acids and their derivatives [Michael *et al.*, 2000]. Transport of amino acids is much easier. The research conducted by Nicklin *et al.* [1995] showed that transport of acidic amino acids is determined by diffusion direction, temperature and pH, and that it is affected by sodium ions. Amino acids are transported by carriers and their transport may proceed even in the direction opposite to the concentration gradient. An important role in the transport of peptides and proteins by Caco-2 cells is played by vitamin B_{12}. The study by Russell-Jones *et al.* [1999] shows that the transport of peptides and proteins is facilitated by creating a complex with cobalamine. At the first stage, a bond is formed between vitamin B_{12} and the protein internal factor; this is followed by binding with a peptide or protein and facilitating the transport. The study results suggest that transport independent of the internal factor is also possible. The manner of transport of proteins depends largely on the concentration of cobalamine on the surface of micro-villi.

The study of intestinal transport with the use of a model of enterocytes *in vitro* includes lipids. In a human alimentary tract, fatty acids exist as micelles with bile salts. Tranchant *et al.* [1997] conducted experiments into explaining mechanisms of the transport of α-linolenic acid (18:3). The fatty acid, dissolved in 10 mmol/L taurocholineate, was taken up on the apical side at a speed which was non-linear and saturative in character. The uptake of acid was greatly affected by the temperature and energy supplied as ATP. The results indicate that the transport of long-chain fatty acids depends on specific carriers with molecular weights of 71 and 88 kDa.

An important role in the transfer of soluble substances is played by bile salts. There are various reports, suggesting that they can reduce absorption by introducing the transported substances to bile micelles, which decreases the thermodynamic activity of these substances. On the other hand, many papers point out that the presence of bile salts favourably affects the transfer of substances as a result of changes in the function of cell membranes and mucin layers and facilitates diffusion in intercellular spaces. Meaney & Driscoll [1999, 2000] conducted a thorough study of the effect of the micellular system on the transport of hydrophilic markers of various molecular weights through a Caco-2 layer. The research included trihydroxy-non-conjugated bile salts, sodium cholineate and sodium taurocholineate. Both simple and mixed micellular systems, composed of bile salts and linoleic acid, were used. Polyethylene glycol and mannitol (molecular weight of 182 kDa) were used as hydrophilic markers. The simple micelles of non-conjugated bile salts and sodium cholineate were found to cause a larger transfer of hydrophilic markers than the non-conjugated bile salts. In the case of sodium cholineate, the increase in the transport speed of hydrophilic substances probably resulted from damage to cell membranes, observed in MTT tests and by TEER measurement. This factor, being strongly polar, also proved to be cytotoxic. The micellular system of sodium taurocholineate was less toxic to enterocytes and significantly facilitated intercellular diffusion. Similarly, conjugates of sodium cholineate with linoleic acid proved more toxic than taurocholineate conjugates. In the case of taurocholineates, not only cellular, but also intercellular transport was possible.

An interesting study on the transport of oligosaccharides from human milk through Caco-2 epithelial cells was described by Gnoth *et al.* [2002]. Human milk contains rare fucosyled oligosaccharides whose structure enables them to act in a way similar to immunomodulating agents and to inhibit inflammations. These oligosaccharides are not digested but they are absorbed from the small intestine. The researchers found that 90-min exposure is followed by oligosaccharides diffusing from the apical side to the base side. The transfer in the opposite direction was also observed. The transfer from the apical side was faster than from the base side for neutral oligosaccharides. No such a relationship was observed for acidic oligosaccharides.

The model of human enterocytes is readily applied in research into the transport of metal ions. One of the main ions absorbed in the large intestine is iron and its transepithelial transport is very important for the total balance of this ion in a human body. Sanchez *et al.* [1996] studied the effect of lactoferrin, transferrin and NO on the transfer of iron ions. They showed that iron is absorbed with the greatest speed after it is ingested as citrate; the absorption is the slowest in the form of the complex with lactoferrin. Caco-2

cells display an ability to express a large number of transferrin receptors; however, the authors postulated that only some products of the degradation of this protein, but not the whole lactoferrin, take part in transporting iron. Introducing nitrogen oxide to the test environment precipitated absorption of iron introduced as citrate. Some interesting research into the absorption of iron ions by Caco-2 cells was conducted by Gangloff *et al.* [1996]. They made a simulation of transferring beef, ascorbic acid and iron citrate through an artificial alimentary tract. In the vessel imitating pancreatic digestion, they placed dialyzing sacs with 12–14 kDa cutoff, which simulated absorption from the small intestine. It was found that the digested beef and ascorbic acid facilitate the transport of iron through a monolayer of enterocytes, whereas ferric citrate was not absorbed so quickly. It was also suggested that there are some agents in the digested meat, which stimulate the absorption of iron.

Another example of applying the Caco-2 line to study the transport of metal ions is the research by Arredondo *et al.* [2000], devoted to copper ions. Under natural conditions, the absorption of copper ions takes place in the initial sections of the small intestine. The researchers introduced the copper-histidine complex, with radioactively-marked $^{64}Cu^{++}$, to a 14-day-old culture of enterocytes. The speed of transfer of copper ions through a monolayer of enterocytes was constant for 1.5 h, after which it rapidly dropped until achieving a state of saturation. At the same time, copper concentration inside the cells was found to increase 21-fold. This means that Caco-2 cells can accumulate large amounts of this metal. The research indicated that the speed of copper transport through an epithelium layer depended largely on the concentration of these ions on both sides of the membrane, which indicates the active control of their transfer. The transporting proteins produced in cytoplasm take part in transporting copper. When the amount of copper is large, the speed of zinc transfer also increases. The transport of both of these ions may be regulated by metalothionine, a protein with many cystein residues. Caco-2 cells were also applied to study cadmium transport [Pigman *et al.*, 1997]. The researchers showed that the infiltration of cadmium through a monolayer of enterocytes proceeds partly by passive diffusion; and that vitamin D_3 and compounds with sulphohydryl groups are partly involved in that process.

Interesting research into the absorption of magnesium was described by Planes *et al.* [2002]. The paper also contains one of the rare descriptions of experiments in which the transported substance, introduced to the medium of Caco-2 culture, was administered not as a pure substance but as a food product, previously digested with enzymes using an alimentary tract imitation (HCl: pepsin and pancreatic enzymes: bile salts). An original method of a transporting experiment was also applied. Shortly before the main experiment, the medium was replaced with MEM without magnesium. In the transporting test, a solution with food products after *in vitro* digestion was used instead of Hanks salts (HBSS). The amount of the product was adjusted so that its 1.5 mL contained 0.17 mg of magnesium. The absorption process lasted 2 h. It was found that the speed of magnesium absorption depended largely on the type of the magnesium-containing product. It was also shown that the absorption of magnesium in the form of chloride was faster than of that from digested food products. When calculating the amount of absorbed magnesium in milligrams of cell proteins and units of introduced magnesium, it may be seen that the differences in absorbing magnesium from different sources were not distinct.

Literature provides reports on attempts to use animal intestinal explants in *in vitro* research into the transport of molecules through a layer of intestinal epithelium. Pietzonka *et al.* [2002] used explants of swine intestines in research into absorbing nanomolecules of a polymer of polyacto-co-glycolic acid and polystyrene through Peyer's crypt tissue and other epithelial tissues. It was found that the amount of absorbed polymers was minute. It was also shown that tissues obtained from a slaughterhouse quickly lose their integrity. Lysis and detaching the epithelial layer, connected with disintegration of the villi structure of intestines, proceeds as soon as after 25 min. The changes account for the failures experienced when intestinal explants were used in research into the transport of polymer molecules through intestinal epithelium.

A comparative study with the use of human colonic explants and a Caco-2 monolayer was carried out by Levy *et al.* [1999]. They proved that mature Caco-2 enterocytes introduce oleic acid to phospholipids, triglycerides and cholesterol at a speed 23 time higher that an intestine section. Significant differences were noted between both biological objects in terms of synthesis and secretion of lipids, apolipoproteins and lipoproteins.

Explants of rat intestine have also been used to study the transport of nucleosides. Iseki *et al.* [1996] used vesicles isolated from the brush border of a rat small intestine. The research covered the transport of mercaptopurine riboside, adenosine and uridine in the presence of Na^+ and K^+ ions. Sodium ions were found to play an important role in this process.

Cell lines of intestinal epithelium in research on carcinogenesis

Cell lines of enterocytes from *in vitro* cultures are also used to study the effect of carcinogenic agents on tumor formation. One of the main causes of carcinogenesis is damage to the DNA structure, caused by mutagens (genotoxic agents). Free radicals are among the most commonly known mutagens and the diet has a decisive effect on the type and amount of produced radicals. A key role in a carcinogenic transformation of cells is played by mutation in genes, which control the growth, and differentiation of cells and which participate in linking extracellular and intracellular signalling to the cellular response. These include pro-oncogens, suppressor genes and protein-encoding genes, which repair errors in damaged DNA. Mutations within these genes, together with defects in chromosomes and cytotoxic effects, usually initiate, promote and favour the progression of malignant tumors.

Genotoxic effects are commonly detected by the COMET method, which consists in electrophoretic analysis of damage in DNA structure of single cells [McKelvey *et al.*, 1993] and by the TUNEL method. Glei *et al.* [2002] used the cell line HT29, clone 19A, to study the induction of oxidative damage of DNA structure when the supply of iron in the diet is high. Enterocytes were cultured in the medium containing ferric-nitrilotriacetate and hydrogen peroxide. It

has been shown that the presence of iron ions induces the cleaving of DNA chains and oxidating of nitrogenous bases in nucleotides. Damaged parts of DNA were detected by the comet assay. It was also found that introducing quercetin as an antioxidant produced a protective effect, and the number of defects was significantly smaller. Similar research was conducted by Kosikova *et al.* [2002], who employed Caco-2 cells. Hydrogen peroxide, which generates hydroxyl radicals, and N-methyl-N'-nitro-N-nitrosoguanidine, which brings about the alkylation of DNA, were used as mutagens. Lignin and its derivatives were used as a protective substance. Defects in the DNA structure were determined by the comet assay.

Numerous scientists use epithelial cell lines to study the proliferation and apoptosis of those cells induced by various factors [Meisel *et al.*, 1998] and to study the activity of enzymes of the I and II phase of carcinogenic modification [Gervot *et al.*, 1996].

Study of immunostimulation

Intestinal enterocytes and Caco-2 cells, stimulated by some agents, display an ability to produce and secrete a range of intestine inflammation cytokins, such as IL-1, IL-6 and IL-8, and chemotactic cytokins for monocytes, such as MCP-1 [Panja *et al.*, 1995]. Rodriquez-Juan *et al.* [2000] studied the secretion of cytokins IL-1, IL-6 and IL-8 and MCP-1 in response to the presence of lectins from leguminous plants. Lectins possess an ability to bind specifically to carbohydrate residues, which are found in many food products. They are often resistant to cooking and enzymatic digestion. Due to this, they are found in the large intestine in an active form. Many lectins are toxic. For example, after ingesting raw or undercooked beans (*Phaseolus vulgaris*), humans fall ill with acute enteritis. It was found that of the above-mentioned interleukins, Caco-2 cells produced only interleukins IL-8 in response to leguminous plant lectins. In response to 100 μg/mL of lectins from *Lens culinaris*, the amount of produced IL-8 increased after 48 h of culturing from 9.2 pg/mL (control sample) to 42.7 pg/mL; for *Vicia faba* the value was 144.8 pg/mL and for *Phaseolus vulgaris* 220.3 pg/mL. It was also noted that the amount of the produced IL-8β, activating the production of IL-8 by Caco-2 [Yang *et al.*, 1997], increased almost 100-fold. This means that lectins start the metabolic path of IL-8 synthesis. It should also be borne in mind that IL-8 are potential chemoattractants for neutrophilia which after being activated become agents in destroying a tissue and taking part in the inflammatory process. It is probable that prolonged administration of lectins may contribute to an inflammation condition in intestines.

CONCLUSIONS

Epithelial cell cultures *in vitro* are a suitable model to study functional properties of foods including probiotic bacteria adhesion, pathogenic bacteria and virus's invasion, gut-associated lymphoid tissue immunostimulation, carcinogenic and mutagenic effects of different substances, and intestinal activity of enzymes. In the review numerous examples of the application of this model for the investigation of biological activity of food compounds are described. However, in future it is necessary to show that epithelial cell cultures cultivated in laboratory conditions have qualities similar to those present during growth under natural conditions in the human alimentary tract.

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FINAL REPORT

Title of the research ordered project:
THE METHODOLOGICAL BASES OF THE EVALUATION OF THE QUALITY AND SAFETY OF THE NEW GENERATION FOODS (PBZ-KBN-020/P06/1999).

Title of the individual project:
The use of the in vitro epithelial cell cultures for the evaluation of probiotic properties of foods.

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Key words:
Caco-2, HT-29, Int 407, *Lactobacillus*, *Bifidobacterium*, lactic acid bacteria, probiotic, adhesion, bioavailability, transport, vitamin B, chromium, atrazine, ferritin, interleukin

SYNTHESIS OF RESULTS

The aim of this work was to estimate the usefulness of epithelial cell cultures *in vitro* as a model to study functional properties of ford compounds. The tests were conducted using three cell lines: Caco-2, HT-29 and Int 407. The cells were cultured according to conventional protocol and using two new methods elaborated in our laboratories: an accelerated method and two-zone anaerobic-aerobic culture. The investigations focused on the parameters influencing culture development, such as serum supplementation, inoculum size, addition of compounds stimulating cell maturation and differentiation, culturing time, mode of aeration and nutrient feeding and culture method.

The epithelial cell monolayer, developed according to these culture methods, was applied to examine bacterial adhesion properties. It was shown that bacterial adherence to intestinal cells is strain- and cell line-dependent. Among cell line tested, the highest affinity to probiotic bacteria was displayed by Caco-2 cells. Comparative study on the use of other surfaces to adhesion study demonstrated their lower usefulness as compared to intestinal cells. Only carboxymethylcellulose membrane gave the results approximate to those obtained with the epithelial models.

The epithelial cell cultures were also used to study the bioavailability of different food compounds as riboflavin, thiamine, pyridoxine, chromium, and atrazine. The studies included bi-directional transport from the apical to the basolateral direction and in reverse. The subjects of our interest were concentration of substance added, exposition time, transport direction, temperature and culture age. The effects of these factors on uptake and transport rate were determined.

The *in vitro* Caco-2 model was applied in the study on mutagenic, carcinogenic and allergenic activity of krezol, phenol, ammonium zearalenol and probiotic bacteria. On the basis of molecular analysis, it was shown that krezol, phenol and ammonium diminished cell viability and caused cell apoptosis. The most destructive was the micotoxin.

The exposition of Caco-2 cells to lactic acid bacteria did not induce the production of interleukin 6 and 8.

In general, the studies presented in this work confirm a versatile usefulness of epithelial cell cultures for the examination of functional properties of food compounds *in vitro*.