Comparative *in vitro* study on the adhesion of probiotic and pathogenic bacteria to different human intestinal cell lines

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Summary

The ability of four strains of *Lactobacillus* sp. two strains of *Bifidobacterium* sp. and one strain of *Listeria monocytogenes* to adhere to human intestinal cell lines Caco-2, HT-29 and Int 407 was examined. Well-developed monolayers of intestinal cells were obtained when initial concentration of Caco-2 cells was $1 \times 10^4/cm^2$, HT-29 cells $4.2 \times 10^4/cm^2$, and Int 407 cells $2 \times 10^4/cm^2$. The appropriate fetal bovine serum additions for Caco-2, HT-29 and Int 407 were 20%, 10% and 10%, respectively. The reduction of serum addition decreased intestinal cell density and prolonged monolayer development. The highest cell densities in epithelial monolayer were obtained in the Int 407 cell cultures. The yield of bacterial adhesion was strain – dependent. Significant differences were also observed in bacteria adhesion to individual intestinal cell lines. The best adhesion ability to Caco-2 exhibited *Lactobacillus rhamnosus* GG and *Bifidobacterium bifidum*. The highest adhesion to HT-29 line demonstrated *B. bifidum* and *Lactobacillus acidophilus* LC1. The adhesion of bacteria to Int 407 was much lower. Significant effect on bacteria adhesion has their cell density being in contact with intestinal monolayer. The adherence of *Listeria monocytogenes* to Caco-2 and HT-29 was very low in the range of 0.2% and 6.0%, respectively.

Key words:
adhesion, probiotic bacteria, intestine cell lines, Caco-2, HT-29, Int 407.

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1. Introduction

The gut microbiota have been implicated as major determinants of health in humans. The probiotic strains stimulate immune system, improve nutrient digestion and bioavailability of different compounds, stimulate bowel motility, possess antimutagenic and anticarcinogenic properties, produce antibacterial factors antagonistic for human enteropathogens and regulate intestine microflora [1-3]. The best documented and longest history of proved health benefits are for the following bacteria strains: *Lactobacillus rhamnosus* GG, *Lactobacillus johnsonii* LJ-1 (LA-1), *Lactobacillus casei* Shirota, *Lactbobacillus acidophilus* LC1, *Lactobacillus reuterii* ATCC 557-30, *Enterococcus faecium* SF68, *Bifidobacterium lactis (bifidum)* Bp-12, and *Saccharomyces boulardii.* One of the main criteria for selecting probiotic strains is their ability to adhere to intestinal surface [4,5]. Attachment to intestine epithelium prolongs the time of maintenance of these bacteria in alimentary tract, which allows to influence human health and microbiota of a host.

It has been estimated that 99% of all bacteria living in natural environments exist in biofilm [6]. Bacterial adhesion is initially based on non-specific physical interactions between two surfaces. They are the attractive van der Waals forces, repulsive electrostatic double-layer forces, hydrophobic interactions short-range forces and ion bridging [7,8]. After attachment to the epithelium surface, the key role play the interactions between bacterial adhesins and complementary epithelial receptors [9-13]. In bacterial adhesion, the principal role play flagella, fimbriae, pili and chaperons. These locomotory organs are coated with complex protein assemblages, including different adhesins which promote bacteria adhesion to intestinal mucin and enterocytes. The type of adhesin depends on the strain, culture factors and receptors expressed by the human enterocytes. The detailed studies on adhesion of *Streptococcus pyogenes* suggest two stage adhesion process: loose reversible binding followed by tight irreversible attachment [14].

Studying bacterial adhesion *in vivo* is difficult, therefore, *in vitro* models with intestinal cell lines are widely adapted for this assessment [15]. The intestinal bacteria are associated with viscous layer of the mucus. It consists of mucin, polypeptide chain, with repetitive amino acids sequences divided by cysteine residues, and carbohydrate moieties. Mucinous proteins are loosely bound to glycocalix overlying the epithelial cell membrane. This complex is produced by epithelial cells, has dynamic fluid state and moves with the intestinal peristalsis. Numerous data suggest the ability of probiotic bacteria to adhere and colonise the intestinal mucosal. Therefore, human intestinal mucus is also used as an additional model for studying bacterial adhesion [16-19]. Epithelial cell lines used in laboratory study possess an ability to produce the glycocalix and mucus. Owing to this, they are an *in vitro* model satisfactorily simulating *in vivo* conditions.

The most common by used epithelial model is enterocyte like cell line Caco-2, established by Fogh et al. [20]. It was isolated from the colon adenocarcinoma of

72-old Caucasian man and possesses an ability of adherent growth on solid surfaces and microporous membranes. During the growth, it undergoes a spontaneous differentiation. After 2-3 weeks of culturing, the cells confluence the culture vessel surface with monolayer of high polarized, enterocyte-like cells, with basolateral localized nuclei, dense mitochondria and brush border with microvilli from luminal side of the cells. After entire confluence of culture surface, the development of cell monolayer is finished. The Caco-2 line is representative for small intestine-type enterocytes and was used for adhesion study by many authors [15,21-25].

Another most commonly used cell line is HT-29, which was isolated from human colon adenocarcinoma. This line is more complex and consists of enterocytes and globlet cells. Because of its ability to produce large quantity of mucus, it is representative for colon epithelium. The best-known mucus forming line is HT-29MTX, a mutant of HT-29. The HT-29 line has a typical morphology of epithelial cells, but does not differentiate into forms with typical brush border. It is widely applied in the studies on intestinal bacteria adherence [19,26].

The third line used in this study is Intestine 407. It is derived from a malignant small intestine of a 2-month old human embryo [27]. This line does not differentiate to a normal polarized cell layer. It is able to secrete a complex extracellular matrix. On the basis of molecular analysis, it was shown that Int 407 possesses some fragments of HeLa's DNA.

The aim of our study was to determine the adherence ability of six strains of probiotic bacteria to Caco-2, HT-29 and Int 407 cell lines. Bacteria attachment was affected by epithelial cell type and this aspect has been discussed.

2. Materials and methods

2.1. Bacteria and culture condition

The strains *Lactobacillus casei* Shirota ATCC 39539, *Lactobacillus acidophilus* LC1, *Lactobacillus rhamnosus* GG ATCC 53103, and *Lactobacilus helveticus, Listeria monocytogenes, Bifidobacterium bifidum* and *Bifidobacterium animalis* from own collection were used in this study. The bacteria were grown anaerobic in MRS broth (Merck, Darmstadt) for 24 h and, afterwards, used for adhesion studies.

2.2. Epithelial cell lines and culture condition

The established cell lines Caco-2 (ATTC HTB 37) and Intestine 407 (ATTC CCL6) were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma), supplemented with 20% (Caco-2) or 10% (Int 407) heat inactivated (56°C, 30 min) fetal bo-

vine serum (FBS, Gibco BRL), 1% non-essential amino acids 100X (NEAA, Sigma) and 50 mg l^{-1} gentamycin (Gibco BRL), whereas HT-29 (ATTC HTB 38) in hybrid Medium Serum Free (Sigma) supplemented with 10% FBS. In the experiments with different serum addition, the medium was supplemented with 4%, 10%, and 20% FBS. The culture medium was changed daily in all experiments. In the experiments with different inoculum size, the wells were seeded with 0.2; 0.4; 1.0; 2.1; 4.2; 6.2 $\times$ 10^4 cells per cm^2 and grew as monolayers for 21 days in 6-well plates (Nunc) at 37°C, in a 5% CO_2/95% air atmosphere. The results presented in this paper are the mean of average vahies and corresponding standard deviations (SD) of three independent experiments.

2.3. Adhesion assay

The bacteria cultures in MRS medium were harvested by centrifugation at 5,600 g for 10 min, washed twice with 0.85% saline and diluted to the standardized concentration of 1 $\times$ 10^6 CFU/cm^3 in DMEM and without any antibiotic.

The 21 day-old Caco-2, 10 day-old HT-29 and 8 day-old Int 407 monolayers, developed in 6-well microplates, were washed twice with PBS. One mililiter of inoculum with defined bacteria concentration was added to each well of the tissue culture plate, and the plates incubated anaerobic at 37°C for 60 min. Afterwards, the DMEM was removed and the monolayers were washed three times with PBS to remove the non-attached bacteria cells. Following the last wash, epithelial cells were gently detached by trypsinisation, harvested by centrifugation, and incubated with 1% Triton X-100 in PBS for 5 min to lyse epithelial cells. The adherent bacteria were counted by plating the serial 10-fold dilution of the suspensions using agar plates. The bacteria strains were cultured anaerobically on MRS agar (Merck). The epithelial cell counts were determined by trypsinisation and enumeration of the cells in Neunbauer haemocytometer in parallel. The results were expressed as a number of bacteria attached to 1000 epithelial cells and to one cm^2 of the cell monolayer surface.

2.4. Scanning electron microscopy

The epithelial cell monolayers, developed on membrane surface, were washed twice with PBS and fixed with 2.5% glutaraldehyde in 0.1 M Millonig's phosphate buffer for 1 h. Afterwards, the samples were dehydrated in a graded series of ethanol, starting with 30%, followed by 50% and 70% solutions. Each wash was repeated twice and lasted 5 min. The samples were coated with gold before the examination on a scanning electron microscope.

2.5. Analytical methods

The enterocyte-like cell concentration was determined using Neunbauer haemocytometer and the cell viability was evaluated by 0,4% trypan blue exclusion dye.

The glucose and lactic acid concentrations were analysed with HPLC method. The proteins containing supernatant in cell culture were precipitated by 5% trichloroacetic acid addition and incubated at 4°C for 1 h. Then, the samples were centrifuged (15 min, 8000 g) and filtered through a 0.22 μm filter (Millex GP, Millipore). The determination of glucose and lactic acid was carried out on MERCK-HITACHI system consisting an autosampler (model L-7250), a pump (model L-7100) and a refractive index detector (model L-7490). The analyses were performed isocritically at a flow rate of 0.8 ml min^{-1} at 30°C, on an Aminex HPX-87H, 300 × 7.8 mm column (Bio-Rad). 0.005 M sulfuric acid was used as a mobile phase. The standard was used to identify the peaks in the chromatograms, and the peak area was used to determine the glucose and lactic acid concentration.

The specific rates of glucose consumption and lactic acid accumulation were calculated from the following equation:

$$q = [(dY/dt) / (dX/dt)]\mu, \text{ where:}$$

Y – glucose/lactic acid concentration, X – viable cell density, t – time, μ – specific growth rate.

The monolayer integrity was determined using the Millicell Electrical Resistance System (Millipore). To monitor the evolution of confluence, the transepithelial electrical resistance (TEER) of Caco-2 cell monolayer was measured daily.

As a measure of cell differentiation, an activity of alkaline phosphatase (ALP) was determined in cell lysates using an Alkaline Phosphatase Kit (POCH, Cat. 178152149). The adherent cells were harvested in cold phosphate buffer saline solution (PBS, pH 7.4) and used for ALP assay.

2.6. Statistical analysis

A Student's t-test was used to examine significant differences ($P<0.05$) in cell growth and adhesion (ANOVA), and the Tukey and Kruskal-Wallis tests to determine the statistical differences between the groups (STATISTICA 6.0. software).

3. Results and discussion

3.1. Characteristics of epithelial cell cultures

In the studies on bacteria adhesion, three intestinal cell lines: Caco-2, HT-29 and Int 407 were used. The appropriate conditions for bacteria adhesion require the use of well-differentiated enterocyte monolayers with a formed brush border and a developed apical cell surface. The critical parameters for enterocyte proliferation is the initial number of cells used for inoculation and fetal bovine serum supplementation.

Figures 1-3 show the growth cycles of individual lines obtained with different initial cell densities. The inoculum size varied in a range from $0.2 \times 10^4/cm^2$ to $6.2 \times 10^4/cm^2$.The results of these experiments indicate that initial cell density significantly influenced the growth kinetics and time necessary for a complete morphological differentiation of cultures. Generally, it can be noted that low densities of inoculum resulted in slow culture development, low maximum cell densities and culture plateau reached not before 12-15 days of culturing. In Caco-2 cultures, the maximum cell density did not exceed $2 \times 10^5 cells/cm^2$ and total surface confluence was not reached when inoculum density was up to $0.4 \times 10^4/cm^2$ (Fig. 1). An increasing seeding to $1 \times 10^4/cm^2$, a significant increase in culture development was observed and culture plateau was shortened by about three hours. Statistically, no significant differences in maximum cell densities were determined between seeding

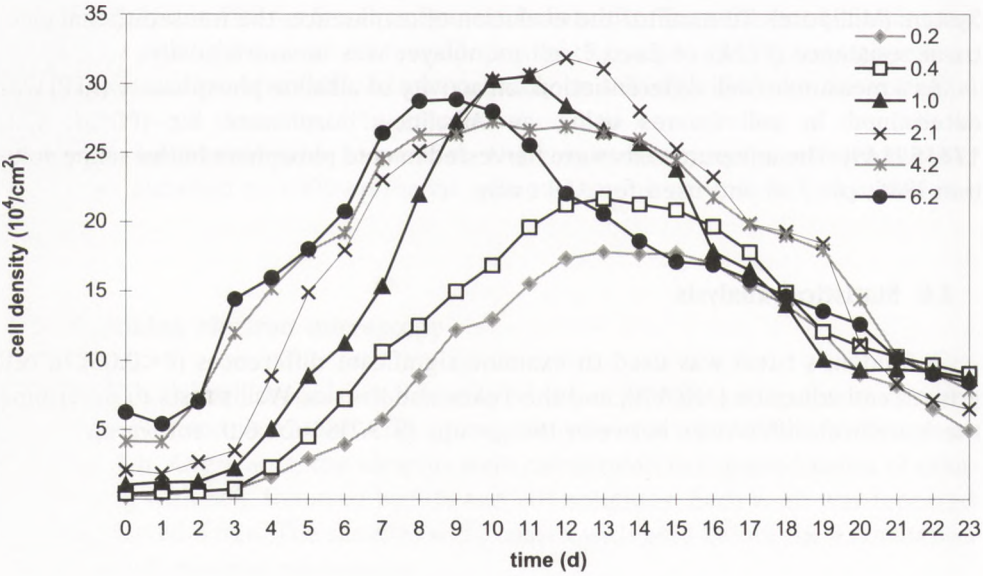

Fig. 1. Influence of initial cell density on the growth kinetics of Caco-2 culture.

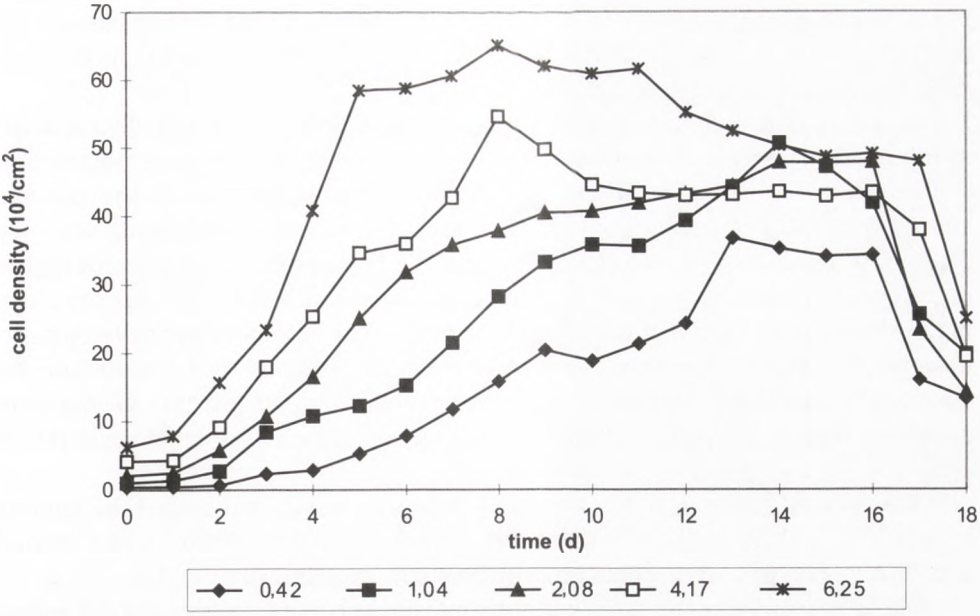

Fig. 2. Influence of initial cell density on the growth kinetics of HT-29 culture.

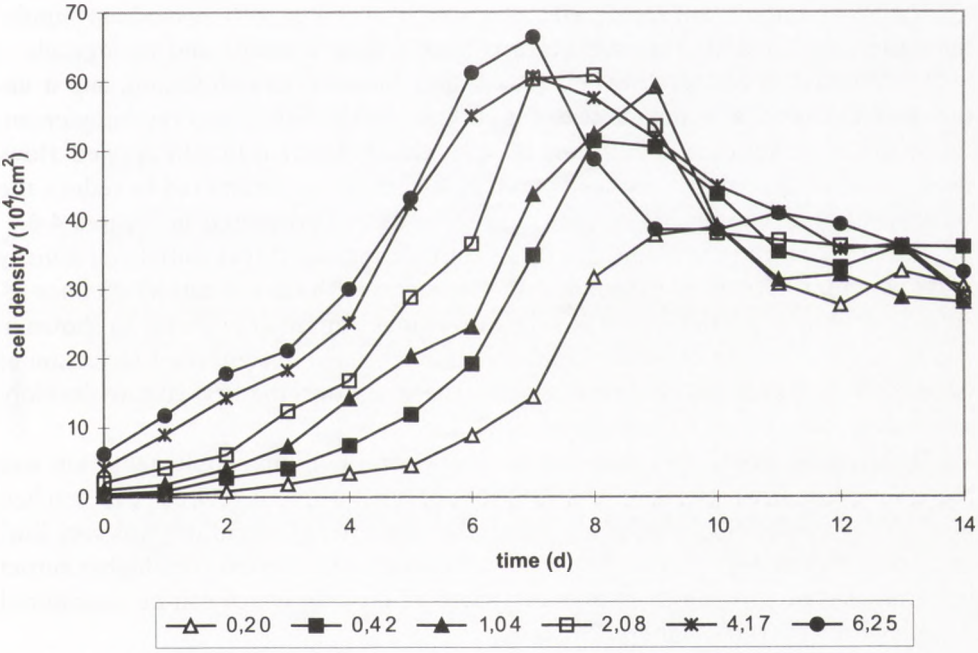

Fig. 3. Influence of initial cell density on the growth kinetics of Int 407 culture.

in the inoculum range from 1 to 6.25 × 10^4/cm^2, however, the culture plateau appeared earlier when inoculum size was higher. The maximum inoculum level shortened the culture plateau to 7-8 days.

The effect of inoculum size on monolayer development was especially apparent in HT-29 cultures (Fig. 2). Statistical analysis shows that cell densities obtained in culture seeded with 0.4 × 10^4/cm^2 significantly differ from those in the cultures started with higher inoculum size. In this case, the maximum cell density was only 36.7 × 10^4 cells/cm^2 and was 70% lower than the best results obtained with higher inoculum. Additionally, the culture plateau appeared not before 13^{th} day of culturing. An increase of inoculum size considerably accelerated monolayer development, however, between the inoculum densities from 1 × 10^4/cm^2 to 4.2 × 10^4/cm^2 no statistically significant differences were determined. Further increase of inoculum density to 6.25 × 10^4/cm^2 resulted in the highest cell densities obtained with HT-29 cells.

Comparing the three cell lines studied, the Intestine 407 presented the highest growth rate and the highest cell densities, reaching 60.9 × 10^4/cm^2 when seeded with 0.2 × 10^4/cm^2, were obtained with this culture (Fig. 3).

Taking into account the doubling time, maximum culture density and cell viability, the optimal inoculum size for Caco-2 was 1 × 10^4/cm^2, for HT-29 4.2 × 10^4/cm^2, and 2 × 10^4/cm^2 for Int 407. These values were applied in the further experiments on bacteria adhesion. Other authors, carrying out investigation on the bacteria adhesion, used similar inoculum concentrations (22,28-30).

The second important factor, affecting epithelial cell growth, is medium supplementation with serum. This substance contains many valuable and biologically – active compounds accelerating cell growth and monolayer proliferation, and it improves enterocyte attachment to solid surface. In the literature, the supplementation of Caco-2 culture with 20% and HT-29 with 10% serum is usually applied. However, these additions are very costly and, in our study, we attempted to reduce the serum addition. The results of these experiments are presented in Figures 4-6.

The analysis of growth kinetics of all cultures indicated that initial cell density plays an important role in monolayer development. Both Caco-2 and HT-29 lines resulted in serum reduction with considerable changes in growth course. As shown in Figure 4, the decrease of serum supplementation caused in significant reduction of Caco-2 cell densities and cell viabilities. For these reasons, the best culture development was achieved at 20% serum addition.

The reaction of HT-29 culture to the reduction of serum supplementation was more complex. The highest cell density was obtained when the culture was reached in 1% of serum addition (Fig. 5). However, the viability of this culture was very low, and optimal monolayer development was obtained at 10% serum. The higher serum level allowed prolonging the stationary phase of growth, which can be considered beneficial for bacterial adherence study.

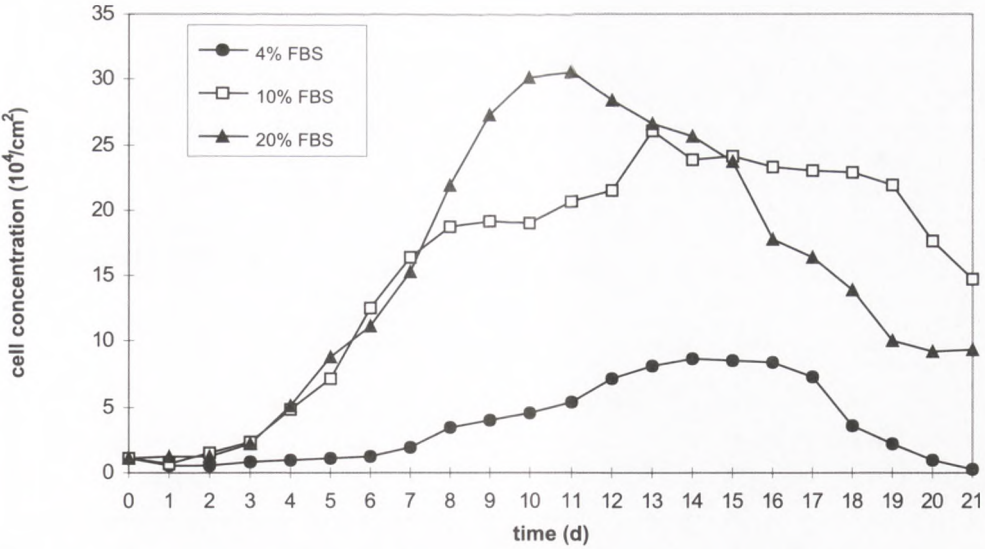

Fig. 4. Influence of fetal bovine serum supplementation on the growth kinetic of Caco-2.

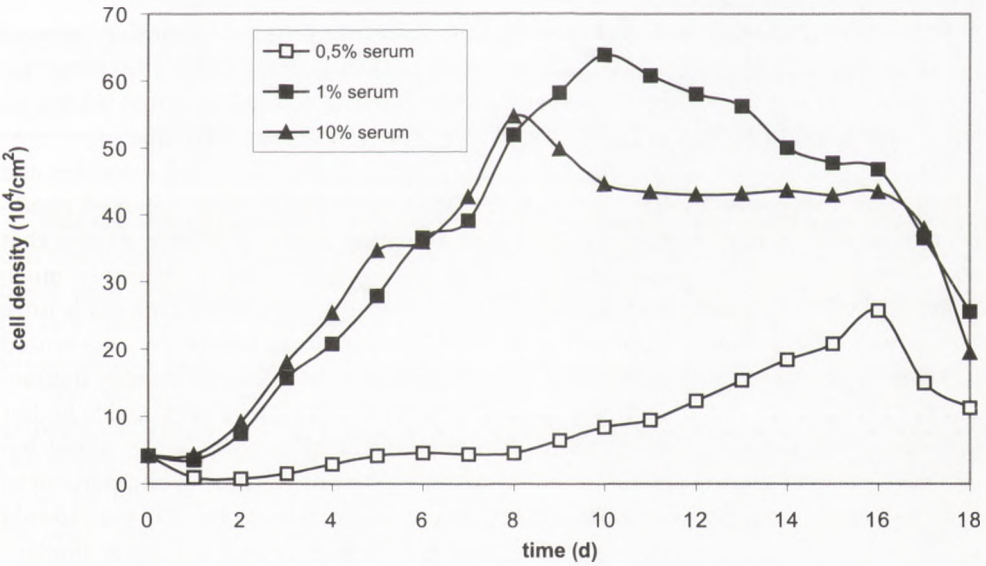

Fig. 5. Influence of fetal bovine serum supplementation on the growth kinetic of HT-29.

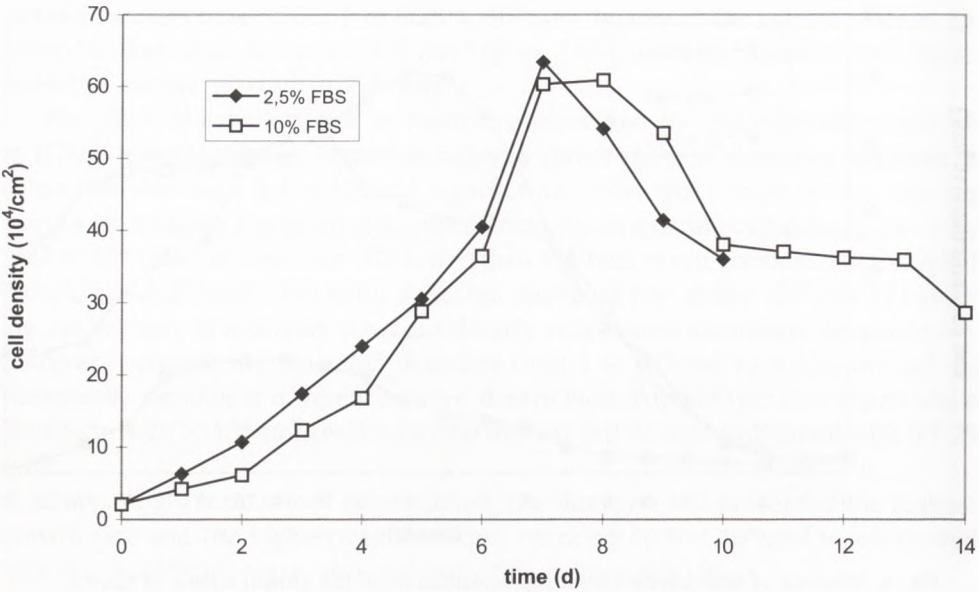

Fig. 6. Influence of fetal bovine serum supplementation on the growth kinetic of Int 407.

The least sensitivity on serum supplementation exhibited Int 407 line (Fig. 6). Comparing 2,5% and 10% serum addition, both serum levels caused similar culture development, however, the higher serum addition prolonged the stationary phase and improved cell viability.

Taking into account the cell growth rate, long duration of stationary growth phase and cell viability our investigation confirmed the data reported by other authors, and for further study on bacteria adherence the following serum additions were applied: 20% for Caco-2, 10% for HT-29, and 10% for Int 407 line.

The studies carried out on the selection of appropriate initial cell densities and serum additions were finalized with the cultures performed at the selected conditions. The time course of epithelial cultures, shown in Figure 7, demonstrated that the fastest growing lines are Int 407 and HT-29, whereas Caco-2 produced much lower cell densities and reached the culture plateau 4 days later than both lines mentioned. These observations are confirmed by the culture parameters presented in Table 1. The shorter doubling time and the highest maximum cell density demonstrated Int 407. The cell densities produced in Int 407 cell culture were twice higher than those obtained in Caco-2 culture. No significant differences were noted between HT-29 and Int 407 cell densities in extreme point of culture. In comparison to both cell lines, the glucose uptake and lactic acid production by Int 407 was 10-fold and 5-7-fold higher, respectively. Caco-2 and HT-29 lines presented many similarities in doubling time and glucose metabolism.

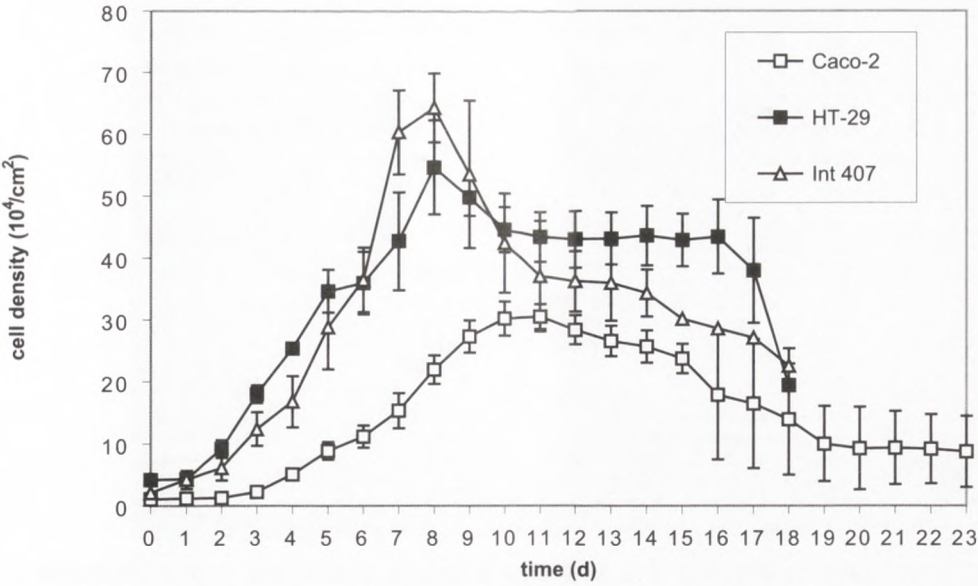

Fig. 7. Time course of Caco-2, HT-29 and Int 407 cultures seeded with optimal initial cell densities.

Table 1

Characteristics of the epithelial cell cultures

Cell line	Doubling time (h)	Cell viability (%)	Maximum cell density (10^6/cm^2)	Glucose uptake (10^{-6}mM cell^{-1} h^{-1})	Lactic acid production (10^{-6}mM cell^{-1} h^{-1})	Glucose: lactic acid ratio
Caco-2	42.5	97	0.305	0.096	0.25	2.6
HT–29	44.5	97	0.546	0.090	0.34	3.8
Int 407	34.7	98	0.609	1.002	1.84	1.8

Interesting data were obtained in the scanning microscopy examination. Caco-2 cell line completed its differentiation during 21 days of culturing and a well-developed monolayer was revealed in the microscopic images. As shown in Figure 8, Caco-2 cells form a homogenous monolayer with visible brush border and single drops of the mucus on their surface.

The microscopic image of Int 407 presented in Figure 10 shows a dense cell growth which resulted in a well-developed monolayer. Similarly to HT-29 (Fig. 9), this line produces relatively great amount of mucus. From morphological point of view, Int 407 monolayer structure resembles Caco-2 line. One aspect of Caco-2 and Int 407 monolayer development is formation of characteristic "cobble-stone" macrostructure, with visible groups of cells forming numerous plate-shape complexes.

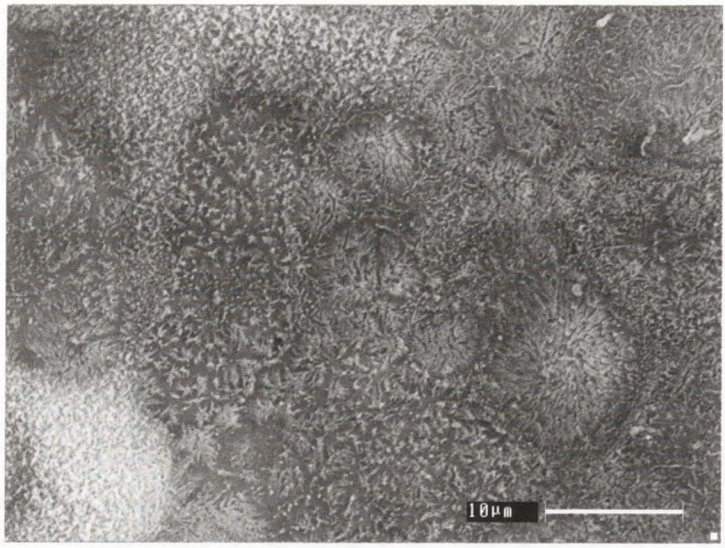

Fig. 8. Caco-2 cells grown in the form of well-differentiated monolayer –3740× (Bar: 2 µm).

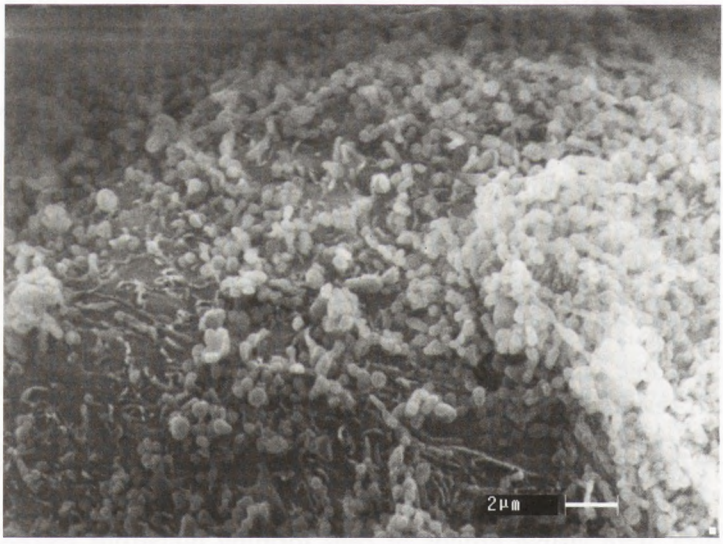

Fig 9. Globlet HT-29 cells surface covered with mucus – 7000× (Bar: 2 µm).

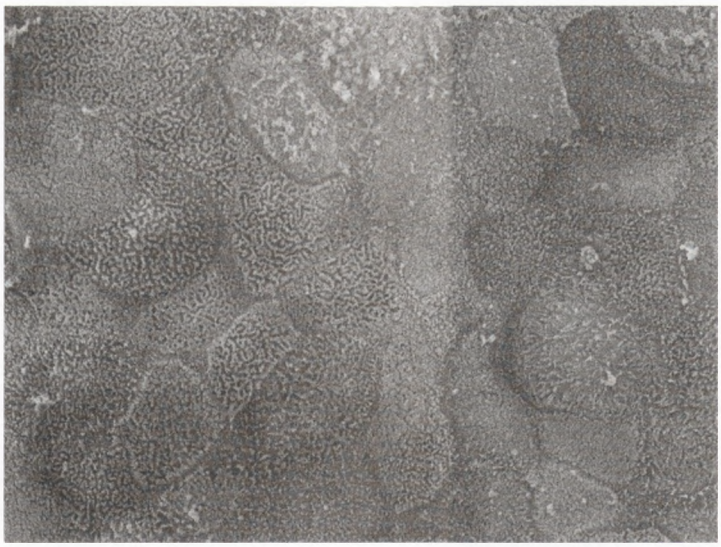

Fig. 10. Well-differentiated Int 407 monolayer covered with mucus in the center – 3140× (Bar: 3 μm).

The monolayer structures obtained in this study seem to be appropriate for bacterial adhesion examination. They possess large surface of microvillar brush border and secrete intestinal mucus, which resembles the *in vivo* epithelium structure.

3.2. Adhesion of bacteria to epithelial cell lines

We selected four strains of *Lactobacillus* and two *Bifidobacterium* strains for the adhesion study. Some of these bacteria are known for their probiotic properties confirmed in clinical trials and well described in the literature. The adhesive abilities of these bacteria are studied *in vitro* by many authors. Usually in these experiments, human epithelial cell lines, mainly Caco-2 line have been used.

There are many reports showing that the adhesiveness, as well as other probiotic properties of lactic acid bacteria, are strain dependent. Nevertheless, there are scarce comparative studies in the literature on bacteria adherence using different human epithelial cell lines for the same bacteria strain examination, which should be helpful for better evaluation of usefulness of enterocyte-like lines for such investigation.

In our experiments, we used intestinal cell cultures at the age corresponding to the plateau of culture, when the cell densities were the highest and monolayer well developed and differentiated.

3.3. Adhesion to Caco-2 cells

The results presented in Table 2 show that all bacteria tested demonstrated an adherence to Caco-2 monolayer. The highest adhesion ability was showed by *L. rhamnosus* GG which was attached to enterocyte-like cells in ratio 1:1. According to statistical analysis of the results, this strain differs significantly from other *Lactobacillus* strains. Other lactobacilli attached to Caco-2 monolayer in quantities about 10-times lower and statistical analysis based on Tukey's test ($P<0.05$) did not detect any significant differences between their adhesive abilities. Comparing both bifidobacteria, *B. bifidum* had significantly higher adhesive properties than *B. animalis*. Taking into account adhesive ability, human pathogenic strain of *Listeria monocytogenes*, which attached only 8.5 cells to one thousand Caco-2 cells and 430 cell per 1cm^2 of monolayer surface, exhibited the lowest adherence. The number of *L. monocytogenes* cells attached to 1 cm^2 was only 0.6% of that shown by *L. rhamnosus* GG, and 1% shown by *B. bifidum*.

Table 2

Parameters characterizing adhesion of bacteria to the Caco-2 cells

Microorganism	Number of bacterial cells attached to 1,000 epithelial cells	Number of bacterial cells attached to 1 cm^2 of epithelium surface ($\times 10^4$ cells)	Adhesion yield (%)
Lactobacillus casei Shirota	94	0.42	2.02
Lactobacillus acidophilus LC1	138	1.03	4.95
Lactobacillus rhamnosus GG	1085	7.43	33.81
Lactobacillus helveticus	112	0.81	3.89
Bifidobacterium bifidum	262	4.38	21.02
Bifidobacterium animalis	55	0.49	2.35
Listeria monocytogenes	8.5	0.043	0.21

Similar conditions for the examination of lactic acid bacteria adherence applied Kankaanpää et al. [22]. These authors reported that adhesion yield of *L. rhamnosus* GG to Caco-2 monolayer amounted 6.0%, *L. casei* Shirota 1.6%, and *L. bulgaricus* 1.9%. Some bacteria strains showed much better adhesion to the mucus isolated from healthy infant's faeces, which amounted 15.8%, 2.8% and 16.1%, respectively. In our study, the adherence of *L. rhamnosus* GG was much higher, whereas the results on *L. casei* Shirota adhesion are similar. The study on the adhesion of *L. casei* Shirota and *L. rhamnosus* GG to Caco-2 monolayer also investigated Lee et al. [28]. They reported that the maximum number of bacteria cells attached to 1,000 Caco-2 cells amounted 1379 for *L. casei* Shirota and 16129 for *L. rhamnosus* GG which is about 10 times higher than that obtained in our study. However, it should be stressed that

these authors used very large inoculum (10^7-10^8 per Lab-Tek chamber slide, Nunc), and they found that adhesion of these strains depended on bacteria density added to Caco-2 culture. The results obtained in this work confirmed that the adhesion of *L. rhamnosus* GG to Caco-2 monolayer is higher than that of the *L. casei* Shirota. According to Gopal et al. [31], adhesion of *L. rhamnosus* GG and *L. acidophilus* LC-1 to Caco-2 reached 1450 and 1840 cells per 1,000 epithelial cells, respectively.

Blum et al. [32] carried out a comparative study on adhesion of different *Lactobacillus* strains to Caco-2 cells. They proved strain dependence on the adhesive properties of bacteria. For example, the results obtained with four *L. acidophilus* strains showed 8.6-fold difference in their adhesion ability.

3.3.1. Adhesion to HT-29 cells

The adherence of bacteria to HT-29 cells was relatively equal and ranged from 14 to 91 cells per 1,000 epithelial cells (Tab. 3). The highest adhesion level showed *L. acidophilus* LC1, but the lowest – *Listeria monocytogenes*. Comparing lactobacilli adherence, statistically significant difference was found only between *L. acidophilus* LC1 and *L. casei* Shirota.

Table 3

Parameters characterizing adhesion of bacteria to the HT-29 cells

Microorganism	Number of bacterial cells attached to 1,000 epithelial cells	Number of bacterial cells attached to 1 cm^2 of epithelium surface ($\times 10^4$ cells)	Adhesion yield (%)
Lactobacillus casei Shirota	28	0.36	12.50
Lactobacillus acidophilus LC1	91	1.20	42.14
Lactobacillus rhamnosus GG	48	0.68	23.69
Lactobacillus helveticus	47	0.80	28.10
Bifidobacterium bifidum	54	1.74	60.93
Bifidobacterium animalis	21	0.74	25.87
Listeria monocytogenes	14	0.17	6.02

Gopal et al. [31], who examined adherence of lactic acid bacteria to HT-29 cells, reported that adhesion yield for *L. rhamnosus* GG amounted 1050 cells per/1000 epithelial cells and 1210 cells/1000 epithelial cells for *L. acidophilus* LC-1. These values are much higher than those obtained in our experiments, but the bacteria densities used by these authors were 100 times higher.

3.3.2. Adhesion to Intestina 407 cells

The adhesive properties of Int 407 were lower than those demonstrated by HT-29 and Caco-2. The number of bacteria attached per 1000 Int 407 cells varied from 4 to 11 (Tab. 4). The highest adhesion to this line exhibited *L. acidophilus* LC1, followed by *L. rhamnosus* GG. The adhesiveness of *L. casei* Shirota nad *B. animalis* was much lower and amounted 4 per 1000 cells of enterocytes. The number of bacteria cells attached to 1 cm^2 of monolayer surface is also similar to HT-29 line. It ranged from 0.28 x $10^4/cm^2$ for *L. casei* Shirota to 0.72 x $10^4/cm^2$ for *B. bifidum*.

Table 4

Parameters characterizing adhesion of bacteria to the Int 407

Microorganism	Number of bacterial cells attached to 1,000 epithelial cells	Number of bacterial cells attached to 1 cm^2 of epithelium surface ($\times 10^4$ cells)	Adhesion yield (%)
Lactobacillus casei Shirota	4	0.28	6.24
Lactobacillus acidophilus LC1	11	0.72	0.98
Lactobacillus rhamnosus GG	8	0.55	4.39
Lactobacillus helveticus	6	0.41	4.22
Bifidobacterium bifidum	5	0.30	5.57
Bifidobacterium animalis	4	0.35	2.86

For assessment of bacterial adhesion to epithelial cell monolayer three parameters are usually applied: the number of bacterial cells attached to 1,000 epithelial cells, number of bacterial cells attached to 1 cm^2 of epithelium monolayer and percentage of bacteria attached to epithelial cells in relation to the bacteria number introduced over monolayer surface. The first parameter depends on the size of epithelial cells and gives only general information. The third parameter is a relative value, not quantitatively ascribing bacterial adhesion. The proper adhesion parameter seems to be the number of bacterial cells attached to a surface unite. In our study, the adhesion of individual strains to each intestinal cell line was differentiated and no simple rules were found in these findings. The Shirota strain showed the best adhesion to Caco-2, followed by HT-29 and Int 407. The difference between the greater and the lower adhesion amounted for about 50%. The strains *L. acidophilus* LC1 preferentially adhered to HT-29 and poorly to Int 407. Taking into account *L. rhamnosus* GG, its adhesion to Caco-2 was about 11 times greater than to HT-29, and 13.5 times greater than Int 407. *L. helveticus* demonstrated similar adherence to Caco-2 and HT-29, and lower to Int 407. The adherence of *B. bifidum* to epithelial cells was high as compared to *Lactobacillus* strains. The adherence of this

strain can be presented in order: Caco-2>HT-29>Int 407. The lowest adhesive properties exhibited *Listeria monocytogenes*.

Many reports showed that bacterial adhesion to the mucus is better than to epithelial cells [16,17,19,33]. Between Ht-29 and Int 407, many similarities were found. Both cell lines demonstrated the highest affinity to *B. bifidum* and *L. acidophilus* LC1 and low affinity to *L. casei* Shirota. The scope of adhesiveness to Caco-2 differs significantly from Int 407. On the basis of this study, the usefulness of Caco-2 line for examination of bacteria adhesion can be evaluated similarly to HT-29, and much better than Int 407. HT-29 and Int 407 lines showed more morphological and physiological similarities to colon epithelium than Caco-2. Moreover, it seems that Caco-2 possess many properties representative for small intestine epithelium and, for this reason, should be more useful for transport studies.

3.3.3. Effect of bacteria density on adhesion

Among important factors influencing bacteria adhesion there is cell density. This aspect of investigation was examined only in relation to *Lactobacillus acidophilus* LC1. In this experiment, two levels of bacteria density were used. The bacteria cells were introduced onto monolayers of Caco-2 and HT-29. The results of this trial, presented in Table 5, revealed that too large seeding of monolayers with bacteria is not efficient. In Caco-2 culture, large bacterial inoculum size decreased the adhesion yields.

Table 5

Characteristics of *Lactobacillus acidophilus* adhesion to Caco-2 and HT-29 epithelial cell lines depending on initial cell densities

Initial bacteria density (10^5/cm^2)	Bacteria attached to 1000 cells	Bacteria attached ($\times 10^4$/cm^2)	Percentage of bacteria attached to the surface (%)
Adhesion to Caco-2 cells			
2.8	151	1.07	3.72
0.9	186	1.84	20.62
Adhesion to HT-29 cells			
1.9	35	0.82	4.17
0.7	31	0.93	13.70

An increase of *L. acidophilus* LC1 density from 0.9×10^5/cm^2 cells to 2.8×10^5/cm^2 caused a decrease of the attached cells from 186 to 151 per 1000 Caco-2 cells, which was reflected in the reduction of the attachment yield from 20.6% to 3.7%. In

the case of HT-29 monolayer, the increase of bacteria density from $0.7 \times 10^5/cm^2$ to $1.9 \times 10^5/cm^2$ caused a decrease of attachment yield from 13.5% to 4.2%.

The effect of bacteria density on adherence efficiency was also examined by other researchers. Lee et al. [28] demonstrated that bacteria cell density had strong effect on *L. rhamnosus* GG attachment to Caco-2 and increased hyperbolically with an increase of bacteria density. The effect of cell density of *L. casei* Shirota on its adhesion to Caco-2 monolayer was much lower, almost negligible. These results are in contrast to our results which demonstrated that decreasing bacteria densities improved bacteria adhesion.

4. Conclusions

1. The time necessary for well development of intestinal cells can be significantly shortened by appropriate inoculation of cultures and supplementation with fetal bovine serum.

2. The yield of bacterial adhesion was strain – dependent and significant differences between the examined strains were observed.

3. The highest number of bacteria was attached to the Caco-2 cell line and the lowest to the Inst 407.

4. Among the examined bacterial strains the highest adhesive properties were exhibited by *Lactobacillus rhamnosus* GG and *Bifidobacterium bifidum*.

5. Human pathogen *Listeria monocytogenes* showed very low adherence to epithelial cell lines.

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