„Determining of adhesive ability of the bacteria contained in combined probiotic preparation to intestinal epithelial Caco-2 cell culture”
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Summary

A growing number of the products (i.e., drugs, dietary supplements) containing various bacterial strains belonging to probiotics can be found on the market. Besides their viability, bacterial adhesion to intestinal epithelium is one of the most important indices of the efficacy of probiotic bacteria-containing preparations.

Aim of work: The aim of the work was to determine the adhesion of the bacteria included in the combined preparation containing 9 different bacterial strains (4 Lactobacillus, 3 Bifidobacterium, 1 Lactococcus, 1 Streptococcus) to Caco-2 intestinal epithelium.

Methodology: Mature, 17-day intestinal epithelial Caco-2 cell culture was used to assess the adhesion ability of probiotic bacteria contained in the studied preparation. Probiotic preparation content (4.5 x 10^9 cells) was suspended in 4.5 ml of saline. Microbial culture was established in MRS medium. Based on the results of previous experiments, we calculated the volumes of cell suspensions containing 5 x 10^6, 5 x 10^7, and 5 x 10^8 cells, respectively.

Calculated parameters: Adhesion rate [%] = (the number of adhering bacteria/the number of bacteria added into a culture) x 100%, the number of bacterial cells bound to the surface of a single intestinal epithelial cell (cfu/single Caco-2 cell, where cfu = colony-forming unit), bacterial dose = the number of bacterial cells used for adhesion per single intestinal epithelial cell (cfu/single Caco-2 cell).

Results: The obtained results indicate that probiotic bacterial strains contained in MULTILAC® preparation have the ability to adhere to intestinal epithelial cells in vitro. The number of bacterial cells bound to Caco-2 cells was dependent upon the preparation dose. The highest adhesion efficacy was observed for the highest inoculum of 5.5 x 10^8 cfu/ml/9.6 cm^2 epithelial tissue in vitro corresponding to a dose of 190 bacterial cells per Caco-2 cell. In this case, approximately 157 bacterial cells underwent adhesion to each intestinal epithelial cell, which corresponded to 83.1 ± 9.5% of the inoculum. Taking into account the criteria published by Candela et al. (2005), it can be said that the combination of microorganisms contained in the combined preparation can be characterized as highly adhesive.

Conclusions: Taking into consideration the literature data characterizing the adhesive properties of single probiotic strains, it can be said that the bacterial strains in the mixture used in combined preparation MULTILAC® exhibit higher efficacy in the colonization of intestinal epithelium. The combination of probiotic bacteria contained in MULTILAC® preparation can be characterized as highly adhesive.
Introduction

In a biological sense, adhesion is defined as a firm, irreversible attachment of microbial cell to the surface of a solid body. Adhesive properties of probiotic bacteria are considered to be the most desirable feature of these microorganisms. Adhesion allows microorganisms and epithelial cells to contact directly, thereby allowing to better counteract pathogenic adhesion. Bacterial high adhesion rate to intestinal epithelium contributes to long-term bacterial residence in the gastrointestinal tract and thereby prolongs the effect on its health. The above-mentioned process occurs even when the bacterial adhesion is transient in nature and does not result in the sustained intestinal colonization [2,10]. The strains demonstrating high adhesiveness are effective in the prevention and alleviation of acute diarrheas. Due to this, apart from the studies on bacterial viability in low-pH and bile- and digestive enzyme-containing environments, studies on bacterial adhesive properties constitute a fundamental stage of research in this area [13,18,19]. The in vitro single-layer epithelial intestinal cell cultures, especially those of Caco-2 and HT-29 cells, are the most frequently used models of bacterial adhesion. Epithelial cultures, which are biologically the closest to intestinal epithelium in vivo, can hardly be considered as an easy investigation model. The maintenance of those cell cultures typically requires specialized equipment; also, it is long-term and very expensive [18].

Material and methodology

Preparation of intestinal epithelial Caco-2 cell culture

Mature 17-day intestinal epithelial Caco-2 cell culture was used to determine the adhesive properties of probiotic bacteria from MULTILAC® preparation. The ampoule with Caco-2 cells (ECACC, 86010202) stored under nitrogen was thawed and then the cells were transferred to DMEM medium (Sigma) with 10% fetal bovine serum (FBS, Gibco) and 1% of a mixture of amino acids (Sigma) with gentamicin (Gibco). Caco-2 cells were cultured in 25-cm² bottles and incubated at 37°C in an atmosphere containing 5% CO₂ and 95% air. The culture was maintained until 80%-90% surface coverage. The cells were then removed from the medium with 0.25% trypsin-versene solution (Sigma) and transferred to a new culture flask. Before the use of the Caco-2 culture, two consecutive 7-day passages were performed. The prepared cells were transferred to a larger culture bottle with growth surface area of 75cm² and the culture was maintained in standard conditions as described above. After 4-day culture, we achieved the cell surface layer covering 80% of the accessible surface of the bottle; that layer was adequate to create an epithelial intestine model to measure the adhesion of probiotic bacteria contained in MULTILAC® preparation. To this end, Caco-2 cells were removed from the flask surface with trypsin-versene solution, cell viability was measured by trypan blue staining, and cell count was determined by hemocytometer method using a Neubauer chamber. The obtained Caco-2 cells were used to establish the cultures intended for the measurement of the adhesion process. The cultures were maintained in 9.5-cm² flasks (Falcon) with initial cell density of 3.4 x 10⁴ cells/cm². Caco-2 cell cultures were incubated for 17 days with the
change of the medium every 24-48h depending on the developmental stage of the culture. Standard culture conditions were maintained as described above. The culture course was monitored by microscopic observations. Before adhesion, the mature layer of Caco-2 cells was washed with phosphate buffer (PBS, Sigma). Before adhesion performance, the number of Caco-2 cells in culturing flask was estimated. That estimation was done by haemocytometer method for three different cultures.

**Preparation of microorganisms to adhesion process**

The MULTILAC® capsule content (4,5 x 10⁹ cells) was suspended in 4.5 ml of saline. The microbial culture was established in liquid MRS medium (10 ml) by adding 100 μl of dissolved MULTILAC® capsule content. The culture was incubated at 37°C for 20h in anaerobic conditions. After the incubation, the microbial culture was centrifuged (4500 rpm, 10h) and the resulting pellet was washed twice with saline.

Based on the results obtained in previous experiments, we calculated the volumes of cell suspensions containing 5x 10⁶, 5 x 10⁷, and 5 x 10⁹ cells, respectively.

**Determination of the adhesion of probiotic bacteria to intestinal epithelial culture**

5x 10⁶, 5 x 10⁷, and 5 x 10⁹ of microbial cells were suspended in 1 ml of pure DMEM medium (without serum and antibiotics) and then added into intestinal endothelium layer. Each sample was done in triplicate. Adhesion process was carried out at 37°C for 90 min. in the atmosphere containing 5% CO₂ and 95% air.

After adhesion, the suspension of bacterial cells non-adhering to epithelium was removed and the epithelium was washed three times with PBS buffer. To release the adhering cells from intestinal epithelial cells, Caco-2 cells were lysed with 1% Triton X-100 solution in PBS. Cell lysis were carried out for 10 min. on ice. Then the lysates were transferred to centrifuge tubes and centrifuged for 10 min. at 4500 rpm. The pellet was washed twice with PBS. The pellet was ultimately suspended in 1ml of saline and the quantitative inoculation aimed at measuring the number of microorganisms adhering to intestinal epithelium in vitro was performed.

The quantitative measurement of microorganisms was performed using the Koch method. Decimal dilutions were made, ranging from 10¹ to 10⁵. The material from each dilution was inoculated into three Petri dishes. The inoculation was made with the pour-plate method using MRS medium. Microbial cultures were incubated at 37°C for 48h in anaerobic conditions. The determination of bacterial cells’ density in the stock culture used in studies of adhesion was also made. After the incubation, the number of colonies was determined and the number of microorganisms adhering to intestinal epithelium was calculated.

Based of the results of the measurements, the bacterial dose used in the determination of adhesive properties, the number of bacteria adhering to epithelium relative to the inoculum used (%), and the number of adhering bacteria relative to the number of epithelial cells were also calculated.

**Results**

All measurements were done in triplicate. The results are summarized in Tables. **Calculated parameters:**
Adhesion rate [%] = (the number of adhering bacteria/the number of bacteria added into Caco-2 cell culture) x 100%,
The number of bacterial cells bound to the surface of a single intestinal epithelial cell (cfu/single Caco-2 cell),

Bacterial dose = the number of bacterial cells used for adhesion per single intestinal epithelial cell (cfu/single Caco-2 cell).

Table 1. Before adhesion

<table>
<thead>
<tr>
<th>Bacterial dose for adhesion/ml</th>
<th>Bacterial dose for adhesion/1 Caco-2 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5.5 \times 10^6$</td>
<td>1.9</td>
</tr>
<tr>
<td>$5.5 \times 10^7$</td>
<td>19</td>
</tr>
<tr>
<td>$5.5 \times 10^8$</td>
<td>190</td>
</tr>
</tbody>
</table>

Table 2. After adhesion

<table>
<thead>
<tr>
<th>Number of adhering cells</th>
<th>% of adhering cells</th>
<th>Number of bacteria bound/1 Caco-2 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2.48 \times 10^6$</td>
<td>45.5 (6.8) %</td>
<td>0.86</td>
</tr>
<tr>
<td>$3.28 \times 10^7$</td>
<td>60.2 (1.7) %</td>
<td>11.3</td>
</tr>
<tr>
<td>$4.53 \times 10^8$</td>
<td>83.1 (9.6) %</td>
<td>156.7</td>
</tr>
</tbody>
</table>
Discussion of results

The obtained results indicate that probiotic bacterial strains contained in MULTILAC® preparation are capable of adhesion to intestinal epithelial cells in vitro. The number of bacterial cells bound to Caco-2 was dependent on the preparation dose. The highest effectiveness of adhesion was seen when the highest inoculum, equivalent to 5.5 x 10^8 cfu/ml/9.6cm² of epithelial tissue in vitro, was used, which corresponded to a dose of 190 bacterial cells per single Caco-2 cell. In this case, 157 bacterial cells underwent adhesion to each intestinal epithelial cell, which corresponded to 83.1 ±9.6% inoculum. Taking into account the criteria published by Candela et al. (3), one can say that the combination of microorganisms contained in MULTILAC® preparation can be characterized as highly adhesive. The authors have classified probiotic microorganisms into three groups: (i) non-adhesive strains when less than 5 bacterial cells undergo adhesion to a single Caco-2 cell, (ii) adhesive strains when adhesion efficacy ranges from 5 to 40 bacterial cells/1 Caco-2 cell and (iii) highly adhesive strains when the adhesion level exceeds 40 bacterial cells bound to 1 epithelial cell (3).

Literature data on the adhesive properties of individual probiotic bacterial strains contained in MULTILAC® preparation show high variability. Depending on the source, origin and dose, various bacterial strains exhibit different adhesiveness to intestinal epithelium. In the case of *Lactobacillus rhamnosus*, adhesion efficacy was estimated at about 7.2-14.4% (22) and 20% (14). Few data point to weak adhesive properties of some *L. rhamnosus* strains, which corresponded to 2 bacterial cells bound to 1 Caco-2 cell (9). Adhesive properties of a single *L. plantarum* strain were defined at the level of 6.7% (22), 80% (14) or expressed as 25 cells bound to 1 Caco-2 cell (3). Comparable adhesion level was the characteristic of both *Bifidobacterium longum* and *Bifidobacterium lactis* genus (about 20 bacteria/Caco-2 cell) (3). Gagnon et al. (2003) found that bacteria of *B. bifidum* and *B. longum* genus underwent adhesion in 3.2-4.2% and 4.6%, respectively, relative to the used dose of 10^7 cfu/well/ml (7). According to Candela et al. (2008), *L. acidophilus* bacteria have a low potential to colonize intestinal epithelium in vitro (about 5 bacterial cells/1 Caco-2 cell) (3).

Conclusions

Taking into account the literature data characterizing the adhesive properties of single probiotic strains, one can say that in MULTILAC® preparation composed of 9 probiotic bacterial strains, individual bacterial strains show higher effectiveness of intestinal epithelium colonization than individual probiotic bacterial strains described in the literature. Probiotic bacterial combination contained in MULTILAC® preparation can be characterized as highly adhesive.