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Real-time PCR quantification of bacterial adhesion to Caco-2 cells: Competition between bifidobacteria and enteropathogens

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Abstract

Probiotic bacteria play an important role in protecting the host from intestinal colonization of pathogenic bacteria. We have developed a new analytical approach based on a real-time PCR technique for quantifying *Bifidobacterium* adhesion to intestinal epithelial cells. Real-time PCR analysis showed that adhesion to enterocyte-like Caco-2 cells represented a variable phenotype in the genus *Bifidobacterium*, enabling classification of three adhesion behaviors: high adhesiveness (>40 bifidobacterial cells/Caco-2 cell); adhesiveness (5–40 bifidobacterial cells/Caco-2 cell); no adhesiveness (<5 bifidobacterial cells/Caco-2 cell). This molecular methodology was successfully used in competition studies in enteropathogens. All bifidobacterial strains examined evidenced displacement activity towards important enteropathogens (*S. typhimurium*, *Y. enterocolitica* and *E. coli* EPEC). Real-time PCR is a rapid, accurate and sensitive method for detecting and quantifying different bacterial genera and species simultaneously adhering to a epithelial cell monolayer.

Keywords: *Bifidobacterium*; Caco-2 cell adhesion; Real-time PCR; Enteropathogenic in vitro competition

1. Introduction

A large population of microorganisms, the so-called microbiota, inhabit the human gastrointestinal tract and form a closely integrated unit with the host. The quantity of living bacteria which compose the human microbiota can range from 10^{11} to 10^{12} CFU/g of luminal content and contain up to 500 different species [10]. Several biochemical, immunological and physiological features of the human host are responses to metabolic activities of the normal microbiota [3,27]. By modulating these host microbiota-associated characteristics and by protecting the host from pathogen colonization, the normal microbiota have a direct impact on human host well-being. In particular, well balanced microbiota play a major role in preventing widespread colonization of enteric pathogens [15]. From the host point of view, such a barrier effect can be considered as one of the main functions exerted by the gut microbiota. Three mechanisms of action are involved in the barrier effect played by the intestinal microflora: (i) prevention of enteropathogenic adhesion to the host enterocytes; (ii) favorable competition with exogenous pathogens for nutrient availability in the gastrointestinal ecological niches; (iii) inhibition of growth of pathogenic bacteria by bacteriocin production and lowering of the pH.

Intestinal disorders, antibiotic treatment, stress, and changes in diet influence the individual microbiota, resulting in their depletion and imbalance [7]. The reduction in the normal microflora has negative effects on human well-being and can be frequently associated with greater host susceptibility to enteropathogenic bacterial infections. In order to overcome problems associated with microflora imbalance, or to generally improve the health of the host, the

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concept of modulating the human microbiota by administration of probiotic bacteria has been established [4,6,16, 20,22,23]. Probiotics are defined as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal balance" [6]. *Bifidobacterium* represents such a probiotic bacterial genus and therefore is widely used in food and pharmaceutical probiotic preparations [12,24–26]. Bifidobacteria represent about 8–10% of the normal adult fecal flora and constitute one of the most important human intestinal microbial groups [2,9,19]. The presence of bifidobacteria in the gastrointestinal tract has been associated with several health-promoting effects such as prevention of diarrheas, amelioration of lactose intolerance and immunomodulation [18,24,25]. Furthermore, a role for *Bifidobacterium* in host infection resistance has been proposed, as in vitro and in vivo studies suggested that some bifidobacterial strains exert an antagonistic activity toward enteropathogens such as *Escherichia coli* EPEC, *Salmonella enterica* serovar Typhimurium and *Yersinia pseudotuberculosis* [1,8]. However, although the genome of *B. longum* has recently been sequenced and annotated [21], there is only incomplete information about bifidobacterial physiology and ecology, and very little is known about the specific mechanisms of direct interactions between bifidobacteria and the host.

In this study we propose a new analytical approach, based on real-time PCR and genus- species-specific primers, for the in vitro evaluation of bacterial adhesion to epithelial cells. In comparison with traditional techniques available (viable counts, radiolabeled bacteria, light and electron microscopy) [32], the analytical approach proposed here is rapid, sensitive and particularly useful for bacterial competition studies. This new molecular approach has been used to study the competition between different bifidobacterial strains belonging to *B. lactis*, *B. bifidum* and *B. longum* species, and three important enteropathogens (*S. enterica* serovar Typhimurium, *Y. enterocolitica* and *E. coli* EPEC) for adhesion to monolayers of enterocyte-like Caco-2 cells.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bifidobacterial strains used in this study were *B. bifidum* S16 and S17, *B. breve* BBSF, *B. lactis* L15 and BI07, *B. animalis* MB254 and *B. longum* E18. *B. bifidum* S16 and S17 were isolated from fecal samples of breast-fed infants and *B. longum* E18 from fecal samples of a healthy adult. *B. lactis* L15 originates from "Neslac" (Nestlé, Switzerland), *B. lactis* BI07 and *B. breve* BBSF from VSL Pharmaceuticals (Gaithesburg, MD, USA), and *B. animalis* MB254 from our collection. The enterotoxigenic human isolate *E. coli* H10407 and the enterotoxigenic bovine *E. coli* B44 (Deneke et al., 1983) were kindly provided by S. Tynkkynen, Valio Ltd., Helsinki, Finland. *Salmonella cholerasuis typhimurium*- and *Yersinia enterocolitica*-type strains were kindly provided by A. Essig, Dept. of Medical Microbiology, University of Ulm, Germany.

All bifidobacteria were grown on De Man–Rogosa–Sharpe (MRS) broth with cysteine (0.5 g/l) at 37 °C under an anaerobic atmosphere (Anaerocult, Merck, Darmstadt, Germany). *E. coli* H10407 and B44 were cultivated at 37 °C aerobically on TY-broth. *S. cholerasuis typhimurium* and *Y. enterocolitica* were cultivated aerobically at 30 °C on BHI-broth.

2.2. Caco-2 cell culture

The epithelial intestinal cell line Caco-2 [17] was employed for the adhesion experiments. Enterocyte-like Caco-2 cells show marked characteristics of human intestinal cells, including the ability to differentiate as well as to polarize and form tight junctions [17]. They were obtained from the Deutsche Sammmlung für Mikroorganismen und Zellkultur (DSMZ, Göttingen, Germany) and grown in Dulbecco's modified Eagle Medium (DMEM) (Gibco^{TM} Invitrogen, Karlsruhe, Germany) supplemented with 20% inactivated (30 min, 56 °C) fetal calf serum (PAA Laboratories, Cölbe, Germany) and 1% non-essential amino acids (Gibco^{TM} Invitrogen), at 37 °C in an atmosphere of 5% CO_2 and 95% air. For the adhesion assays, cells were seeded at a concentration of 1×10^5 cells/well in 24-well tissue culture plates (Falcon multiwell^{TM} 24 well, Beckton Dickinson, Sparks, MD). The cell culture medium (2 ml/well) was changed every 2 days and 24 h before an adhesion assay. Cells were used for adhesion assays at late postconfluence, i.e., after 15–17 days in culture and complete differentiation. The viable cell number, counted in a Neubauer chamber, was about 6×10^5 cells per well.

2.3. Radioactive and non-radioactive adhesion assays

Bacterial adhesion to Caco-2 cells was evaluated either by using radiolabeled bacteria and counting Caco-2-bound radioactivity (radioactive adhesion assay), or by quantification of Caco-2-bound bacteria with genus- or species-specific primers via real-time PCR (non-radioactive adhesion assay). All experiments were made at least twice in triplicate.

For radioactive adhesion assays, late exponential cultures of the respective bacteria were adjusted with sterile culture medium to an optical density at 600 nm (OD_{600}) corresponding to 1×10^8 cells/ml. 4.5 ml of this suspension was incubated at 37 °C for 30 min in the presence of 50 µCi [^{35}S] methionine (Amersham Pharmacia Biotech, Germany). After removal of the excess radioactivity by washing four times with sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) (PBS), the bacteria were resuspended in 4.5 ml DMEM and used for the adhesion assays.

The Caco-2 cell monolayers at the bottom of the wells in the tissue culture plates (see above) were washed with DMEM and 1 ml DMEM with 1×10^8 radiolabeled bacteria was added, corresponding to approximately 170 bacteria per Caco-2 cell. After incubation for 1 h at 37 °C in 5% CO_2 and 95% air, unattached bacteria were removed by washing the monolayers four times with sterile PBS. After detachment of the Caco-2 cells from the plastic surface by incubation with 200 µl trypsin/EDTA (PAA Laboratories) per well (10 min, 37 °C), the cells (Caco-2 cells and adhesive bacteria) were transferred to a counting vial containing 100 µl 5% (w/v) SDS in 0.5 M NaOH. The wells were rinsed with 200 µl PBS which was then also transferred to the counting vial. After vigorous mixing, cells were lysed during incubation for 15 min at room temperature. After addition of 4 ml of scintillator Ultima Gold (Packard Biosciences, Dreieich, Germany), vigorous mixing and a further incubation for 1 h at room temperature, the radioactivity was determined by liquid scintillation. The adhesion (given as percentage) was calculated from the ratio between the radioactivity bound to the washed and lysed Caco-2 cells and the total radioactivity of all bacteria added at the start of the experiment.

For non-radioactive adhesion assays, the bacterial cultures were also adjusted with sterile culture medium to an OD_{600} corresponding to about 1×10^8 cells/ml. After washing the bacteria twice with sterile PBS, they were resuspended in DMEM and used for the adhesion assay.

Non-radioactive adhesion assays were performed according to the procedure described above for radioactive adhesion assays, except that unlabeled bacteria were added and, after detachment from the plastic surface, the Caco-2 cells and the adhesive bacteria were transferred to a 1.5 ml-reaction tube. The wells were rinsed with 200 µl sterile PBS which was also transferred to the 1.5 ml reaction tube. The suspensions then were frozen and stored at −20 °C until quantification of the bacteria by real-time PCR. For reference purposes (100% values), 1 ml aliquots of the original bacterial cell suspensions used in the adhesion assay were centrifuged, the cells resuspended in 200 µl trypsin/EDTA plus 200 µl PBS and then frozen and stored at −20 °C until quantification of the bacteria.

For competition assays, suspensions of the selected bacterial strains were prepared as described above (about 1×10^8 bacteria/ml). Caco-2 cell monolayers were incubated with the first strain for 1 h at 37 °C in 5% CO_2, washed with 2 ml DMEM and afterwards incubated with the second strain (1 h, 37 °C, 5% CO_2). In the displacement assay, the first strain was the pathogen and the second the *Bifidobacterium*, whereas in the exclusion assay, the first strain was the *Bifidobacterium* and the second the enteropathogen. Detachment and preparation of the samples for non-radioactive quantification were done as described above. For the 100% values (references), aliquots of each strain used were prepared and quantified separately.

2.4. Quantification of bacterial cells by real-time PCR

For quantification of bacterial cells by real-time PCR, cell suspensions obtained from non-radioactive adhesion assays were thawed at room temperature and, after mixing, an aliquot of 20 µl was transferred into a 0.2 ml-reaction tube and incubated for 10 min at room temperature with 3.8 µl of trypsin inhibitor solution (Type I-S: from soybean, Sigma; 1 mg/ml in H_2O). Then the bacterial cells (*Bifidobacterium*, *E. coli*, *Salmonella* and *Yersinia)* were specifically quantified by real-time PCR performed with the genus- or species-specific primers listed in Table 1.

Real-time PCR was performed in a LightCycler instrument (Roche, Mannheim, Germany) and SYBER Green I fluorophore was used to correlate the amount of PCR product with the fluorescent signal. Amplification was carried out in a 20 µl final volume containing 2 µl of cell suspension, 4 mM of $MgCl_2$, 0.5 µM of each primer and 2 µl of LightCycler-FastStart DNA Master SYBR Green I (Roche). The experimental protocol consisted of the following programs: (i) starting preincubation at 95 °C for 10 min; (ii) amplification including 30 cycles of 4 steps each at the temperature transition time of 20 °C/s: denaturation at 95 °C for 15 s, annealing at the appropriate temperature (Table 1) for 25 s, extension at 72 °C for 30 s, and fluorescence acquisition at the appropriate temperature (Table 1) for 5 s; (iii) melting curve analysis: heating at 20 °C/s to 95 °C; cooling at 20 °C/s to 60 °C with 15 s hold, and then heating 0.1 °C/s until 99 °C. As internal standards we amplified serial dilutions of the respective bacteria in PBS ranging from 1×10^6 to 1×10^3 CFU/µl.

3. Results

3.1. Evaluation of Bifidobacterium adhesion to Caco-2 cells by real-time PCR

Caco-2 cell adhesion activity of 7 bifidobacterial strains belonging to the human species *B. bifidum*, *B. breve* and *B. longum* and the dairy species *B. lactis* and *B. animalis* was evaluated by a real-time PCR-based method (Fig. 1). Non-adhesive *E. coli* B44 and adhesive *E. coli* H10407 strains were used as negative and positive controls, respectively [31]. An extremely variable phenotype of adhesion could be observed with the different bifidobacteria. *B. lactis* BI07 and *B. animalis* MB245 were the most adhesive strains (5.85×10^3 and 5.45×10^3 bacterial cells/100 Caco-2 cells), *B. bifidum* S17 and S16 and *B. lactis* L15 showed an intermediate adhesion activity (3.8×10^3, 3.38×10^3 and 3.3×10^3 bacterial cells/100 Caco-2 cells), and *B. longum* E18 and *B. breve* BBSF displayed the lowest adhesion capacity (1.94×10^2 and 1.77×10^2 bacterial cells/100 Caco-2 cells). On the basis of these results, we defined as non-adhesive bifidobacterial strains with less than 5 bacterial cells adhering to one

Table 1
Genus- and species-specific primers used for quantification of bacterial cells in adhesion assays and their specific annealing and fluorescence acquisition temperatures

Primer	Sequence	Specificity	Annealing temperature (°C)	Fluorescence acquisition temperature (°C)	Reference
Bif 164	5′-CATCCGGCATTACCACCC-3′	*Bifidobacterium*	60	90	[13,33]
Bif 662	5′-CCACCGTTACACCGGGAA-3′	*Bifidobacterium*	60	90	[13,33]
MINf	5′-ACGGTAACAGGAAGCAG-3′	*Salmonella enterica*	55	85	[29]
MINr	5′-TATTAACCACAACACCT-3′	*Salmonella enterica*	55	85	[29]
Y1	5′-AATACCGCATAACGTCTTCG-3′	*Yersinia enterocolitica*	63	85	[35]
Y2	5′-CTTCTTCTGCGAGTAACGTC-3′	*Yersinia enterocolitica*	63	85	[35]
ECO-1	5′-GACCTCGGTTTAGTTCACAGA-3′	*Escherichia coli*	60	88	[34]
ECO-2	5′-CACACGCTGACGCTGACCA-3′	*Escherichia coli*	60	88	[34]

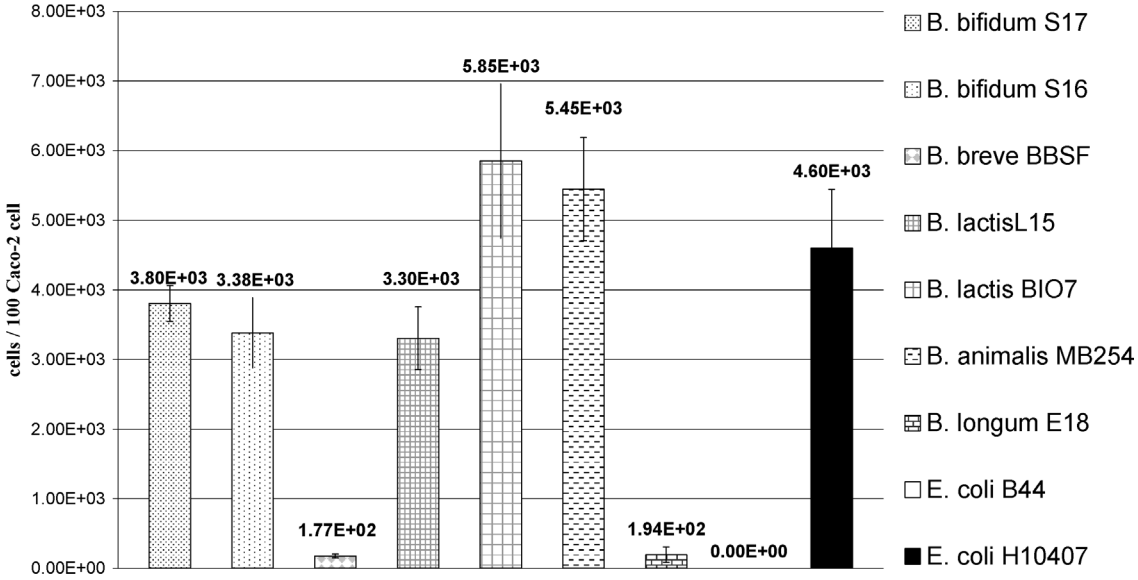

Fig. 1. Adhesion of different *Bifidobacterium* strains and controls, the non-adhesive *E. coli* B44 and the adhesive *E. coli* H1040, to the Caco-2 cell monolayer, as evaluated by real-time PCR. The numbers given above the columns represent means ± standard deviation.

Caco-2 cell, as adhesive the strains with 5–40 bacteria adhering to one Caco-2 cell, and as strongly adhesive those strains with more than 40 bacteria adhering to one Caco-2 cell.

In order to validate bacterial adhesion values obtained by real-time PCR analysis, adhesion of the seven bifidobacteria and the *E. coli* controls was evaluated using the traditional approach performed with radiolabeled bacteria (radioactive adhesion assay). The bifidobacterial adhesion patterns obtained using this traditional method (Fig. 2) were in agreement ($P > 0.05$) with those achieved by real-time PCR analysis for the following strains: *B. bifidum* S17 and S16, *B. breve* BBSF, *B. lactis* L15 and *B. longum* E18. Significant divergences ($P < 0.01$) were found for the adherent strains *B. lactis* BI07 and *B. animalis* MB254, which showed higher adhesion values using the real-time PCR analysis.

3.2. Study of competition between bifidobacteria and enteropathogens for adhesion to Caco-2 cells

To study competition between different bifidobacteria and adhesive enteropathogenic bacteria *S. enterica* serovar Typhimurium, *Y. enterocolitica* and *E. coli* H10407 for adhesion to Caco-2 cells, the real-time PCR-based adhesion assay was employed. On the bases of adhesion scores obtained using real-time PCR (Fig. 1), we tested the strongly adhesive *B. lactis* BI07, the adhesive *B. bifidum* S17 and S16 and the non-adhesive *B. longum* E18. For each bacterial couple *Bifidobacterium* (B)–enteropathogen (P) two competition conditions were performed: (i) a displacement assay (P/B assay), in which the enteropathogen was added to the Caco-2 cell monolayer before the addition of *Bifidobacterium*; (ii) an exclusion assay (B/P assay), in which bifidobacteria were added to the Caco-2 cell monolayer before the addition of the enteropathogen. At the end of both competition assays, *Bifidobacterium* and enteropathogen cells bound to Caco-2 cells were specifically quantified using real-time PCR with genus-species-specific primers. Adhesion values obtained for each strain under competition conditions were compared with those achieved with single strains in adhesion tests performed using an analogous incubation time (S assay).

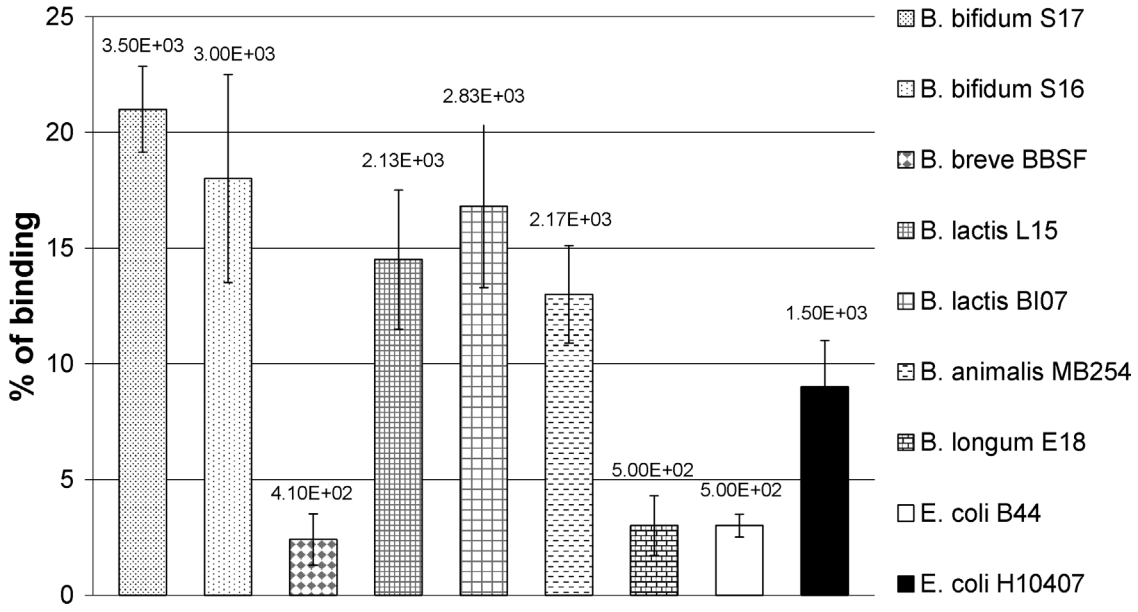

Fig. 2. Adhesion of different *Bifidobacterium* strains and controls, the non-adhesive *E. coli* B44 and the adhesive *E. coli* H10407, to the Caco-2 cell monolayer as evaluated by use of radiolabeled bacteria and counting Caco-2 cell-bound radioactivity. Adhesion is given as the percentage of all bacteria bound to the Caco-2 cells. The values represent the means ± standard deviation. The numbers above the columns represent the means ± standard deviation bound to 100 Caco-2 cells as calculated from the percentage of binding.

In Fig. 3 data regarding the displacement of *S. enterica* serovar Typhimurium, *Y. enterocolitica*, and *Escherichia coli* H10407 from the Caco-2 cell monolayer exerted by *B. bifidum* S16, *B. bifidum* S17, *B. lactis* BI07 and *B. longum* E18 are shown. The adhesion values obtained in assays with single strains are shown as well. The ability of bifidobacterial strains to displace enteropathogens from the Caco-2 cell monolayer was evaluated by calculating the percent reduction of adhering enteropathogen cells in the displacement assay compared to that obtained in assays with solely the enteropathogen. Strong displacement activity towards *S. enterica* serovar Typhimurium and *Y. enterocolitica* was observed with all *Bifidobacterium* strains tested. In particular, the bifidobacteria displaced 97–99% and 75–80% of *Salmonella* and *Y. enterocolitica* cells, respectively. In contrast, bifidobacterial displacement towards *E. coli* H10407 was species-dependent. *B. bifidum* S16 and S17 displaced 69 and 65% of the enteropathogen cells, whereas *B. lactis* BI07 and *B. longum* E18 reached 86 and 76% values. Finally, it is noteworthy that all adhesive bifidobacterial strains were able to significantly adhere to the Caco-2 cell monolayer already colonized by enteropathogens. For instance, *B. bifidum* strains and *B. lactis* BI07 showed adhesion values higher than 10^3 bacterial cells/100 Caco-2 cells when cultured on monolayers previously colonized by *S. enterica* serovar Typhimurium, *Y. enterocolitica*, and *E. coli* H10407.

Fig. 4 shows data on the exclusion of *S. enterica* serovar Typhimurium, *Y. enterocolitica*, and *E. coli* H10407 from Caco-2 cell monolayers previously incubated with *B. bifidum* S16, *B. bifidum* S17, *B. lactis* BI07 and *B. longum* E18. For each strain the adhesion values obtained in assays containing the single strain are also shown. The ability of the bifidobacteria to exclude enteropathogens from Caco-2 cells was evaluated by calculating the percentage of reduction of enteropathogen adhesion values with respect to those obtained in assays with solely the pathogen. Adherent strain *B. bifidum* S16 was the only one to exert a significant exclusion effect towards *S. enterica* serovar Typhimurium, as shown by 39% reduction in adhesion of the pathogen to Caco-2 cells. Adhesive bifidobacteria, but not the non-adhesive E18 strain, showed a significant exclusion effect on *Y. enterocolitica*. In particular, *B. bifidum* S16 and S17, and *B. lactis* BI07 reduced the adhesion ability of *Y. enterocolitica* by 34, 52, and 68%, respectively. None of the bifidobacterial strains tested were able to exert an exclusion effect towards *E. coli* H10407 from the Caco-2 cell monolayer. In the exclusion assays *B. lactis* BI07, *B. bifidum* S16 and *B. longum* E18 showed a strong increase in the number of adhering cells when compared to assays containing only the *Bifidobacterium* (Fig. 4). In particular, *B. lactis* BI07 showed an increase in adhering cells of about 120, 270, and 500% in the exclusion assays with *S. enterica* serovar Typhimurium, *Y. enterocolitica* and *E. coli* H10407, respectively. Similar behavior was observed with *B. bifidum* S16 and *B. longum* E18, with increases in the adherent cells of between 90 and 400%.

4. Discussion

This study reports for the first time the use of a real-time PCR-based method for evaluating bacterial adhesiveness to human epithelial cells. The ability to adhere to the intestinal epithelium represents a significant prerequisite for the

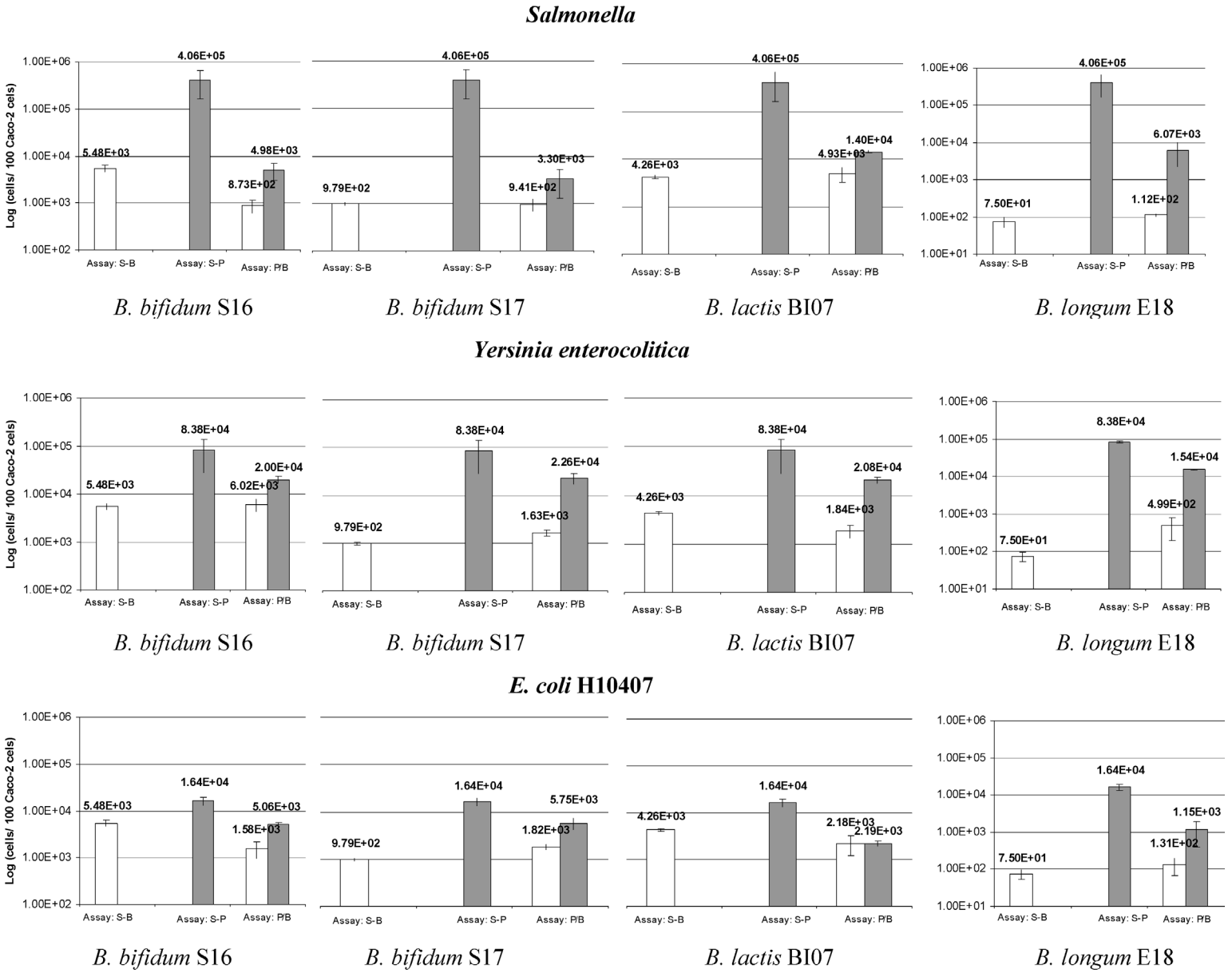

Fig. 3. Displacement assays performed with adhesive *B. bifidum* S16, *B. bifidum* S17 and *B. lactis* BI07 and non-adhesive *B. longum* E18 (B) with *S. enterica* serovar Typhimurium, *Y. enterocolitica*, and *E. coli* H10407 (P). For each couple P + B, the log values of bacteria bound to 100 Caco-2 cells as obtained in assays containing single strains (assays S–B and S–P) and displacement assays (assay P/B), are given. Error bars represent ± standard deviation of mean values. Gray columns represent the pathogen, and white columns the respective *Bifidobacterium*.

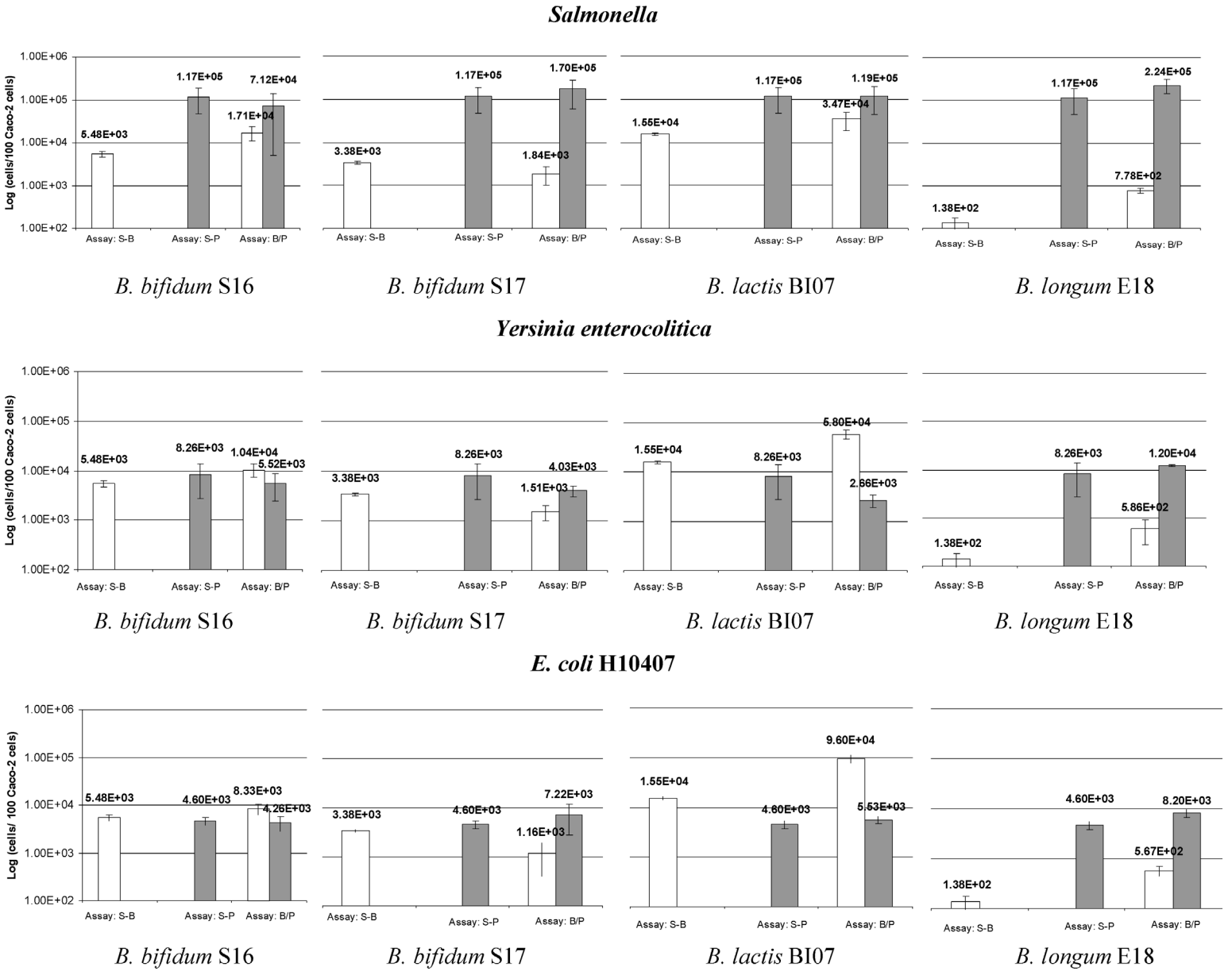

Fig. 4. Exclusion assays performed with adhesive *B. bifidum* S16, *B. bifidum* S17 and *B. lactis* BI07 and non-adhesive *B. longum* E18 (B) with *S. enterica* serovar Typhimurium, *Y. enterocolitica*, and *E. coli* H10407 (P). For each couple B + P the log values of bacteria bound to 100 Caco-2 cells, as obtained in assays containing single strains (assays S–B and S–P) and exclusion assays (assay B/P), are given. Error bars represent ± standard deviation of the mean values. Gray columns represent the pathogen, and white columns the respective *Bifidobacterium*.

transient intestinal colonization of probiotic bacteria [30]. In this perspective, a rapid, accurate and sensitive method for studying bacterial adhesion can be useful for the selection of probiotic bacterial strains. Provided specific primers for a given organism are known, our method allows the quantification of this organism's cell number adhering to a eukaryotic cell. A further important advantage of the real-time PCR-based method is its efficacy in detecting and quantifying different bacterial genera and species simultaneously adhering to an epithelial cell monolayer.

As a first step in this study, adhesion of seven *Bifidobacterium* strains and two *E. coli* control strains was evaluated by means of the real-time PCR approach and the traditional technique employing radiolabeled bacteria. A satisfactory agreement in the bacterial adhesion patterns obtained with the two methods demonstrates the reliability of the new molecular approach here proposed. Real-time PCR analysis showed that adhesion to Caco-2 cells represents a variable phenotype in the genus *Bifidobacterium*, enabling classification into three adhesion behaviors: high adhesiveness (>40 bifidobacterial cells/Caco-2 cell); adhesiveness (5–40 bifidobacterial cells/Caco-2 cell); no adhesiveness (<5 bifidobacterial cells/Caco-2 cell). The adhesion range proposed here is extremely different from that suggested by Del Re et al. [5], who studied adhesion to Caco-2 cells of the *B. longum* species by microscopy counting. Even though Del Re et al. proposed three adhesion phenotypes as well, they report adhesion scores about 100 times lower than those obtained in this study: >40 bacteria/100 Caco-2 cells as strongly adhesive, 6 to 40 bacteria/100 Caco-2 cells as adhesive, and <5 bacteria/100 Caco-2 cells as non-adhesive [5]. According to their classification, strongly adhesive *B. longum* strains adhere with less than one bifidobacterial cell/Caco-2 cell. These data are in clear contrast with results reported by Bernet et al. [1] on the adhesion ability of *Bifidobacterium*. Those authors estimated adhesion of *B. breve* and *B. infantis* strains to Caco-2 cells by scanning electron microscopy and referred to the presence of a kind of biofilm constituted by bifidobacteria adherent to each other on the Caco-2 cell surface. These observations support the adhesion range here proposed for classifying bifidobacterial adhesiveness. The variability of the adhesion phenotype shown in the *Bifidobacterium* genus supports the usefulness of real-time PCR analysis for rapid high-throughput adhesion testing of probiotic strains.

In order to provide insights into the possible role played by resident bifidobacteria in protecting against, or recovery from, pathogen colonization, the competition of different bifidobacteria (*B. lactis* BI07, *B. bifidum* S17 and S16, and *B. longum* E18) and adhesive enteropathogens (*S. enterica* serovar Typhimurium, *Y. enterocolitica*, and *E. coli* H10407) for adhesion to Caco-2 cells was studied. For each couple *Bifidobacterium*–enteropathogen, two competition conditions were tested: displacement and exclusion.

In displacement assays, cells of the enteropathogens were allowed to adhere to the Caco-2 cell monolayer and subsequently the bifidobacterial strains were added. These experiments provide information about the capacity of *Bifidobacterium* to displace the respective enteropathogen from Caco-2 cells and to adhere to an enterocyte monolayer already colonized by the enteropathogen. All bifidobacterial strains examined exerted strong displacement activity towards *S. enterica* serovar Typhimurium and *Y. enterocolitica*, and significant activity towards *E. coli* H1040, including the non-adhesive *B. longum* E18 strain. The latter result suggests that the displacement activity exerted by bifidobacteria towards enteropathogens might also be related to mechanisms other than mere competition for common adhesion sites, such as production of antimicrobial compounds or anti-adhesion factors. This hypothesis is corroborated by Lievin et al. [14], who demonstrated that *Bifidobacterium* strains isolated from infants produce antibacterial lipophilic factor(s) effective in inhibiting *S. enterica* serovar Typhimurium invasion of Caco-2 cells and in killing intracellular enteropathogenic cells. Moreover, Fujiwara et al. [8] reported a proteinaceous factor which in vitro inhibits adherence of an enterotoxigenic *E. coli* strain to gangliotetraosylceramide molecules which are physiological constituents of the mammalian intestinal epithelium surface [11,28].

In exclusion studies, bifidobacterial strains were allowed to adhere to the Caco-2 cell monolayer and then the enteropathogens were added. This in vitro model has been designed to evaluate the capacity of bifidobacteria to prevent enteropathogen adhesion. Different responses were obtained by challenging *Bifidobacterium* with enteropathogens. While all adhesive bifidobacterial strains excluded *Y. enterocolitica*, only *B. bifidum* S16 exerted exclusion activity towards *S. enterica* serovar Typhimurium, and no bifidobacteria strain excluded *E. coli* H1040. Since, in the exclusion assay, anaerobic bifidobacteria are aerobically incubated on Caco-2 cells for more than 1 h before pathogen addition, strong reduction in their metabolic activity might occur. Under these experimental conditions, the ability of bifidobacterial strains to prevent adhesion of *Y. enterocolitica* and *S. enterica* serovar Typhimurium to Caco-2 cells might simply be attributed to competition for common adhesion sites or, eventually, to steric hindrance. However, in view of the reduced metabolic activity of *Bifidobacterium*, an in vivo exclusion effect towards *E. coli*, dependent upon the production of anti-adhesion factors, cannot be excluded.

It is noteworthy that *Bifidobacterium* strains were never displaced by enteropathogens in exclusion assays, and addition of pathogens even induced an increase in the *B. bifidum* S16, *B. lactis* BI0 and *B. longum* E18 cell number on the monolayer. Fast oxygen consumption by the added pathogens (which are administered at a high concentration of 1×10^8 cells/ml) might have favored the growth of anaerobic bifidobacteria.

In conclusion, the real-time PCR-based method proposed here is a useful tool for evaluating the in vitro adhesiveness of probiotic strains and their ability to compete with pathogens for epithelial monolayer adhesion.

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