RESEARCH ARTICLE

Immunization of mice with *Lactobacillus casei* expressing intimin fragments produces antibodies able to inhibit the adhesion of enteropathogenic *Escherichia coli* to cultivated epithelial cells

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Received 12 June 2008; revised 29 July 2008; accepted 31 July 2008.
First published online 17 September 2008.

DOI:10.1111/j.1574-695X.2008.00471.x

Editor: Richard Marconi

Keywords
enteropathogenic *Escherichia coli* (EPEC); intimin; *Lactobacillus casei*.

Abstract

Enteropathogenic *Escherichia coli* (EPEC) are frequently isolated as a cause of infantile diarrhea in developing countries. Its pathogenicity is distinguished by histopathological alterations at the site of infection, known as attaching and effacing (A/E) lesions, in which bacterial virulence factors and host proteins participate. Intimin, a bacterial adhesin expressed by all EPEC described to date, is responsible for the intimate adherence of the bacteria to host cells and is essential for the formation of A/E lesions. Mucosal vaccination may represent an efficacious intervention to prevent EPEC infection and lower morbidity and mortality rates. Strategies for mucosal vaccinations that use lactic acid bacteria for the delivery of heterologous antigens rely on their safety profile and ability to stimulate the immune system. In the present work, we have constructed *Lactobacillus casei* strains expressing different fragments of intimin β, a subtype that is frequently expressed by EPEC strains. Mucosal immunization of mice with *L. casei* expressing intimin fragments induced specific systemic and mucosal antibodies. These antibodies were able to recognize native intimin on the surface of EPEC and to inhibit *in vitro* EPEC binding to epithelial cells.

Introduction

Diarrheal diseases are common pathologies in the childhood. They can be of particular importance when occurring in developing countries, where they are responsible for around 21% of total child mortality (Kosek *et al.*, 2003). Among the pathogens responsible for these infections, enteropathogenic *Escherichia coli* (EPEC) are frequently isolated from infantile diarrhea (Nataro & Kaper, 1998). In Brazil, EPEC may account for 8–43% of diarrhea cases (Gomes *et al.*, 1991; Rosa *et al.*, 1998; Scaletsky *et al.*, 2002; Nunes *et al.*, 2003; Regua-Mangia *et al.*, 2004). When the clinical evolution of this pathology does not cause death, it is responsible for disabilities that impair school performance as well as fitness and cognitive development (Bittencourt *et al.*, 1993; Fagundes-Neto & de Andrade, 1999; Guerrant *et al.*, 2002). These bacteria exert their pathogenicity by attaching to enterocytes and subverting cell signaling, with consequent alteration in cell morphology [the so-called attaching and effacing (A/E) lesions] and physiology and characteristic loss of water (Kaper *et al.*, 2004). EPEC infections can be prevented by breastfeeding (Palmeira *et al.*, 2007) and maintaining good sanitary conditions, as well as good alimentary and hygiene habits (Sobel *et al.*, 2004), which may be a problem in poor regions. Thus, the development of effective vaccines to prevent EPEC infections can be particularly interesting for developing countries.

EPEC can be divided into typical and atypical groups depending on the expression of the bundle-forming pilus encoded by the EAF plasmid (Trabulsi *et al.*, 2002). Nevertheless, a common virulence factor of these bacteria is intimin, a 94-kDa adhesin coded by the *eae* gene, responsible for intimate adhesion of the bacteria to epithelial cells (Kaper *et al.*, 2004). During infection, intimin binds to its

receptor Tir, which is expressed and translocated to host-cell membranes by the bacteria, through a type three secretion system (Kenny, 2002; Dean *et al.*, 2005, 2006). Both *eae* and *tir* genes are located in the locus of enterocyte effacement (LEE), a pathogenicity island present in the EPEC genome and thought to have been acquired by horizontal gene transfer (McDaniel *et al.*, 1995). The intimin molecule presents a conserved N-terminal region, followed by a more variable region that is exposed on the bacterial surface and is therefore in contact with the immune system (McGraw *et al.*, 1999; Tarr & Whittam, 2002). Several intimin subtypes were already described on the basis of the variability of the last 280 amino acids (aa) present in the C-terminal region (Ito *et al.*, 2007). These subtypes have been related to specific host and tissue tropism (Mundy *et al.*, 2007). In addition, this region contains immunogenic epitopes and a binding site to the Tir receptor (Frankel *et al.*, 1995; Adu-Bobie *et al.*, 1998; Batchelor *et al.*, 2000). Despite this variability, intimin β is one of the most frequent subtypes found in EPEC isolates from humans and animals (Penteado *et al.*, 2002; Nunes *et al.*, 2003; Ramachandran *et al.*, 2003; Nakazato *et al.*, 2004; Blanco *et al.*, 2005). The importance of intimin to EPEC virulence has been shown through nonvirulent intimin-negative mutants (Frankel *et al.*, 1996; Cleary *et al.*, 2004; Miyake *et al.*, 2005). Anti-intimin antibodies can be detected in sera and saliva from infected individuals or infants living in endemic areas (Carbonare *et al.*, 2003), as well as in human milk and colostrums (Loureiro *et al.*, 1998). Moreover, immunoglobulin fractions from human milk and colostrums are able to inhibit *in vitro* EPEC adhesion to epithelial cells (Cravioto *et al.*, 1991; Camara *et al.*, 1994). All these considerations suggest that intimin may be a good candidate for vaccine formulations against EPEC infections.

Effective mucosal vaccines represent one of the goals of researchers working in this field, mainly when vaccination is to be carried out in developing countries (Oliveira *et al.*, 2007). This can be explained by the ease of application and the low risk of contaminations, which contribute to lower costs of mass vaccination when compared with parenteral vaccines (Kane, 1998; Oliveira *et al.*, 2007). Still, most of the vaccines that are currently in use are applied through parenteral routes. Lactic acid bacteria (LAB) have been used successfully as live vaccine vectors, carrying different antigens, in animal experimentation (Grangette *et al.*, 2004; Bermudez-Humaran *et al.*, 2005; Oliveira *et al.*, 2006; Hanniffy *et al.*, 2007; Campos *et al.*, 2008). The main advantages of LAB-based strategies are with respect to safety and acceptability related to habitual consumption of these bacteria by populations (Detmer & Glenting, 2006; Wells & Mercenier, 2008). Besides protection of the antigen to be presented on mucosal surfaces, these bacteria also modulate the immune system, working as adjuvants (Mohamadzadeh *et al.*, 2005; Galdeano & Perdigon, 2006). In the present work, we have engineered *Lactobacillus casei* to express fragments of intimin β. Induction of specific antibodies in mice immunized with the recombinant bacteria as well as the ability of these antibodies to bind to the EPEC surface and inhibit adhesion to epithelial cells *in vitro* were analyzed.

Materials and methods

Bacterial strains and growth conditions

LAB were grown in M17 (Difco) containing 0.5% of glucose at 30 °C (*Lactococcus lactis* MG1363) or in MRS (Difco) at 37 °C (*L. casei* CECT5275). The EPEC HSJ-34 (serotype O111ab:H2), expressing intimin β (Campos *et al.*, 1994), and the prototype EPEC E2348/69 (serotype O127:H6), expressing intimin α (Levine *et al.*, 1978), were grown in Luria–Bertani broth (LB) (Difco) or in Dulbecco's Modified Eagle's Medium (DMEM) (Cultilab) depending on the experiment. All bacterial stocks were maintained at −80 °C in their respective culture media, containing 20% glycerol.

Plasmids and recombinant DNA procedures

The pT1NX vector (Campos *et al.*, 2008) was used for constitutive intracellular expression. Fragments composed of a conserved and a variable portion (cv) or only a variable (v) portion of the β intimin gene (Fig. 1) were amplified by PCR, using the EPEC O111ab:H2 genomic DNA and the following primers: Int_{cv} forward: 5′-G ATA TCA GCT AGC AAG TTG CAG TCG-3′ and Int_{cv} reverse: 5′-GGA TCC CTA AGA TCT AGC ATC AAC AGA AGC AAT-3′ or Int_{v} forward: 5′-G CCG GCG ATA TCT ATT ACT GAG ATT AAG GCT G-3′ and Int_{v} reverse: 5′-GGA TCC CTA AGA TCT AGC ATC AAC AGA AGC AAT-3′. These primers were based on the sequence of β intimin (GenBank accession number: AF081187), and were designed in order to include sites for EcoRV and BamHI on the 5′ and the 3′ termini of the amplified fragment, respectively. The PCR conditions were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and 72 °C for 7 min. After sequencing confirmation, the fragments were digested with EcoRV and BamH1 for ligation in the pT1NX vector digested previously with the same enzymes. Competent *L. lactis* and electroporation was performed as described previously (Oliveira *et al.*, 2006). Plasmids isolated from *L. lactis* were then used for electroporation of *L. casei* (Oliveira *et al.*, 2006). *Lactococcus lactis* [Correction added on 25 September 2008: *Lactobacillus* changed to *Lactococcus*] and *L. casei* transformants were selected by plating on the respective media containing 1.8% agar and 5 μg mL^{-1} of erythromycin.

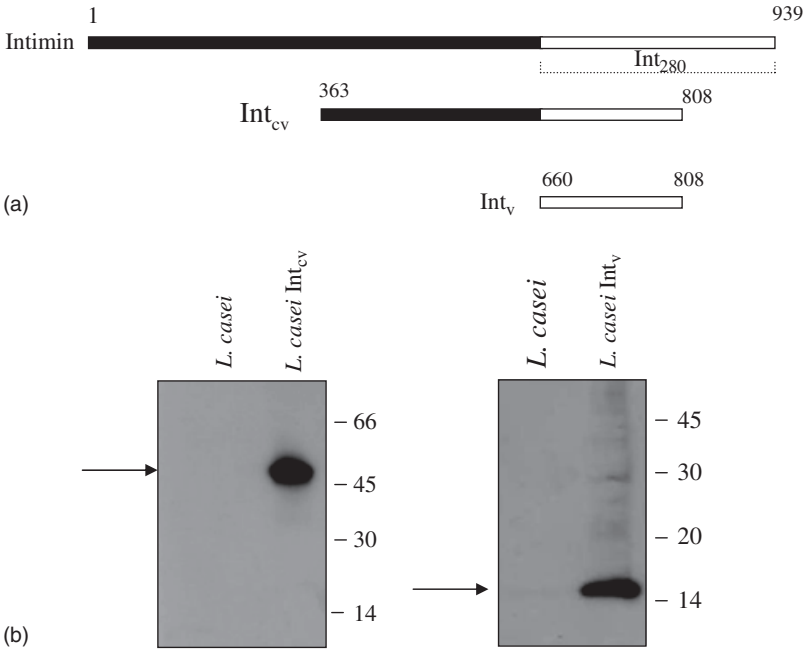

Fig. 1. Expression of intimin fragments by *Lactobacillus casei*. (a) Schematic representation of intimin β and the fragments. Int_{cv} comprises the region from 363 to 808 aa and Int_v comprises the region from 660 to 808 aa. (b) Lysates from *L. casei*-Int_{cv} or *L. casei*-Int_v were separated using SDS-PAGE and transferred to nitrocellulose membranes that were incubated with anti-Int_{cv} antibodies generated in mice immunized with a recombinant *Escherichia coli*-produced Int_{cv} polypeptide. Lysates from *L. casei* were used as negative control. Arrows indicate the Int_{cv} and Int_v bands.

Protein expression and Western blot analysis

Extracts of recombinant *L. casei* cultivated until the stationary phase were prepared by mechanic lysis as described previously (Oliveira *et al.*, 2006), and proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to nitrocellulose membranes, expression of Int_v and Int_{cv} was evaluated by immunoblotting using mouse polyclonal anti-Int_{cv} (produced in our laboratory) and the Enhanced Chemiluminescence developing kit (GE Healthcare). The same protocol was used for immunoblot analysis of O111ab:H2 extracts, prepared from the bacteria cultivated in DMEM or LB until the stationary phase, using 1 : 500 dilutions of sera collected from mice immunized as described below.

Mice immunization

Female C57Bl/6 mice (5–7 weeks old, five per group) from the Central Animal Facility of the Instituto Butantan were supplied with food and water *ad libitum*. Animal experimental protocols were approved by the Ethics Committee for Animal Use of the same Institute. Mice were immunized through the intranasal route with the recombinant *L. casei* expressing Int_{cv} or Int_v (*L. casei*-Int_{cv} or *L. casei*-Int_v), the respective control bacteria carrying the empty vector (*L. casei*) or saline. Briefly, bacteria were grown until the stationary phase ($OD_{550\,nm} > 2.0$), collected by centrifugation (16 000 ***g***, 6 min at room temperature), washed twice and suspended to 10^9 viable cells in 15 μL of saline. Mice were anesthetized through the intraperitoneal route with 200 μL of a 0.5% xilazine and 0.2% ketamine mixture and the cell suspension was inoculated into their nostrils on days 0, 1, 14, 15, 28 and 29. Fifteen days after the last dose, mice were bled by the retrorbital plexus. Nasal washes were also collected 15 days after the last immunization, as described elsewhere (Oliveira *et al.*, 2006). Antibodies were evaluated by an enzyme-linked immunosorbent assay (ELISA), using *E. coli*-produced recombinant Int_{cv} as coating and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-mouse IgA (Sigma Chemical). Differences in antibody titers were analyzed using the Mann–Whitney *U* test, and $P \leq 0.05$ was considered to be significantly different. Titers were defined as the last dilution in which $A_{492\,nm}$ reached 0.1.

Anti-Int_{cv} and anti-Int_v antibodies binding to the EPEC surface

EPEC O111ab:H2 was cultivated in LB for 18 h. The culture was diluted to an $OD_{600\,nm}$ 0.3 in conditioned medium from HEp-2 cell cultures and maintained at 37 °C for 4 h. Bacteria were collected by centrifugation at 5000 ***g*** for 4 min, washed once with phosphate-buffered saline (PBS) and the concentration was adjusted to 10^7 cells per 100 μL. Pooled sera of each immunization group were added to 100 μL of bacterial suspension at 10% (v/v) final concentration. Samples were incubated at 37 °C for 30 min. Bacteria were washed once with PBS and incubated with a 1 : 1000 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) in PBS for 30 min on ice. Samples were washed twice with PBS and stored at 4 °C in 200 μL of 2% formaldehyde, before analysis. As control, bacteria were incubated with PBS, followed by incubation with FITC-

conjugated antibodies. Flow cytometry analysis was conducted using FACSCalibur (Becton Dickinson), and 10 000 gated events were recorded. The percentages of fluorescent bacteria (brighter than 10 fluorescence intensity units) were calculated for each sample.

EPEC adhesion and inhibition assay

HEp-2 cells were cultivated in DMEM supplemented with 10% fetal calf serum (FCS) (Cultilab) at 37 °C in 5% CO_2 (v/v). For the assays, 5×10^4 cells were plated onto glass coverslips inserted to 24-well plates and cultivated for 2 days under the same conditions. A stationary-phase culture of EPEC O111ab:H2 (cultivated for 16 h at 37 °C in LB) was diluted in LB to an $OD_{600\,nm}$ of 0.3. This suspension (2×10^6 bacteria in 25 μL) was incubated for 1 h at room temperature with a 1 : 50 dilution of each serum in a final volume of 1 mL of DMEM containing 2% FCS and 1% D-mannose (VETEC). HEp-2 cells' medium was removed and samples were added to the plates and maintained for 4 h at 37 °C. After 4 washings with PBS, samples were fixed with 70% methanol for 45 min at room temperature. Slides were stained with May Grünwald (Merck) for 5 min and Giemsa (Merck) for 20 min. The percentages of cells displaying adhered EPEC microcolonies were determined. Photographs were taken using the DFC300FXs camera (Leica) and the Axioskop microscope (Zeiss).

For the determination of total bacteria adhered to HEp-2 cells, the same experiment was performed using EPEC O111ab:H2 or E2348/69. After washings with PBS, plates were incubated with 100 μL per well of 1% Triton X-100 for 30 min at room temperature. This suspension was plated in LB and the CFUs were determined after incubation at 37 °C for 18 h. For competition assays, the different sera were incubated with 4 μg of recombinant Int_{cv} before incubation with EPEC, followed by the same protocol.

Results

Expression of Int_{cv} and Int_v by *L. casei*

DNA fragments coding for Int_{cv} (1338 bp) or Int_v (444 bp) were amplified using PCR from the genomic DNA of EPEC HSJ-34 strain (serotype O111ab:H2) (Campos *et al.*, 1994). Sequencing analysis (GenBank accession number EU816360) revealed a 100% identity with the nucleotides from the same region of the EPEC Dec12a strain *eae* gene (GenBank accession number: AF081187). Fragments were cloned into the pT1NX vector (Campos *et al.*, 2008) to allow constitutive expression under the control of the P1 promoter. Plasmids were constructed in *L. lactis* and, after confirmation of the correct cloning, they were used for transformation of *L. casei*. Int_{cv} comprises parts of the conserved and the variable regions of intimin β (from 363 to 808 aa), whereas the Int_v fragment corresponds to part of the intimin β variable region from 660 to 808 aa) (Fig. 1a). Expression of both fragments was confirmed by immunoblots of bacterial extracts using anti-Int_{cv} antisera. As observed in Fig. 1b, the antisera specifically recognized bands of 48 and 15 kDa corresponding to Int_{cv} and Int_v, respectively. The amount of protein expressed by 10^9 lactobacilli was estimated to be around 70–90 ng for both Int_{cv} and Int_v, based on the concentration curves of the respective purified proteins, used as references (data not shown).

Induction of antibodies by nasal immunization of mice with recombinant *L. casei*

C57Bl/6 mice were immunized through the nasal route with *L. casei* expressing Int_{cv} (*L. casei*-Int_{cv}) or *L. casei* expressing Int_v (*L. casei*-Int_v). As controls, mice were immunized with *L. casei* bearing the empty vector (*L. casei*) or with saline. A significant induction of specific IgG was observed in the sera of mice immunized with *L. casei*-Int_{cv} or *L. casei*-Int_v when compared with the group that received saline ($P < 0.05$) (Fig. 2a). Surprisingly, significant levels of specific antibodies were also observed in the group of mice that were immunized with *L. casei* ($P < 0.05$ compared with the saline group). Nasal washes from immunized mice displayed a similar profile of sIgA induction. All animal groups immunized with *L. casei*, including the control bearing the empty vector, presented significant levels of sIgA that recognize the Int_{cv} fragment (Fig. 2b, $P < 0.05$). Evaluation of the preimmune sera collected from individual mice was performed but no reactivity with Int_{cv} was observed (data not shown), discarding the presence of anti-intimin antibodies before immunization. Moreover, nasal immunization of mice with nonrecombinant *L. casei* produced a similar reactivity in sera and nasal samples (data not shown), suggesting that the results observed were not related to the pT1NX plasmid.

Antibodies raised by nasal immunization of mice with the recombinant *L. casei* recognize recombinant Int_{cv} as well as EPEC intimin β

Because the unexpected cross-reactivity of the sera and nasal wash samples from mice immunized with *L. casei* was reproducible, we decided to analyze the recognition of intimin β by immunoblot. Sera from mice immunized with *L. casei*-Int_{cv} specifically recognize a band of 94 kDa, corresponding to the size of the entire intimin β in protein extracts from EPEC O111ab:H2 cultivated in LB (L) or DMEM (D). These sera also recognized Int_{cv} produced in *E. coli* (Fig. 3a, right panel). The same result was observed when the membrane was incubated with sera from mice immunized with *L. casei* (Fig. 3a, middle panel), confirming

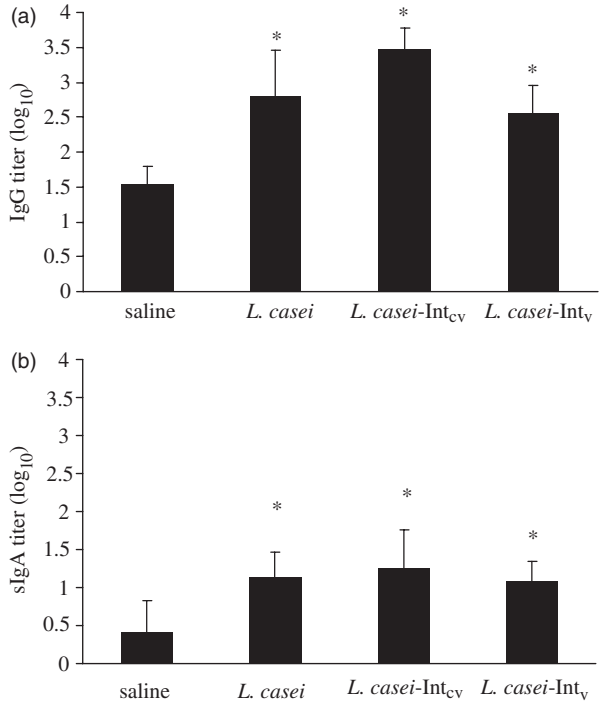

Fig. 2. Induction of antibodies by nasal immunization of mice with recombinant *Lactobacillus casei*. IgG in the sera (a) and sIgA in nasal washes (b) were determined using ELISA against *Escherichia coli*-produced recombinant Int$_{cv}$. Results are represented as means of antibody titers with SDs. Titers were defined as the dilutions in which the absorbances reached 0.1. Zero in the *Y*-axis represents nondetectable reactions in the minimal dilutions used. *Significantly different from saline, using the MannWhitney *U* test, $P < 0.05$. Results are representative of two independent experiments.

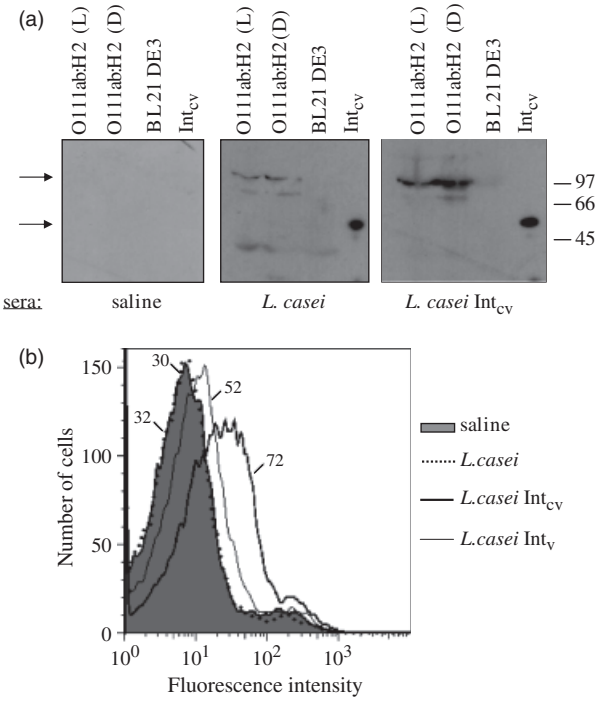

Fig. 3. Recognition of native intimin by sera from mice immunized with recombinant *Lactobacillus casei*. (a) Lysates from EPEC O111ab:H2 cultivated in LB (L) or DMEM (D) were analyzed using SDS-PAGE and transferred to nitrocellulose membranes that were incubated with different sera, as marked. Lysates from *Escherichia coli* BL21 (DE3) and purified *E. coli*-produced recombinant Int$_{cv}$ were used as negative and positive controls, respectively. The arrows indicate the intimin or the recombinant Int$_{cv}$ bands. (b) EPEC O111ab:H2 (10^7 bacteria) incubated with the different sera were washed and further incubated with anti-mouse IgG-FITC. Flow cytometry analyses were conducted with 10 000 gated events recorded. Percentages of fluorescent bacteria (brighter than 10 fluorescence intensity units) are shown for each sample.

the reactivity of these sera with intimin. No reactivity was observed when extracts of *E. coli* BL21 (DE3) were probed with both sera (Fig. 3a, middle and right panels). The sera from mice inoculated with saline did not display any reactivity (Fig. 3a, left panel).

Intimin β in the EPEC surface is only recognized by sera from mice immunized with *L. casei*-Int$_{cv}$ and *L. casei*-Int$_{v}$

In order to evaluate the ability of the different sera to recognize native intimin β and therefore bind to the surface of EPEC O111ab:H2, we incubated them with whole bacteria. Samples were then incubated with anti-mouse IgG conjugated with FITC and analyzed using fluorescence-activated cell sorting (FACS). As shown in Fig. 3b, a positive reaction was observed only for the sera from mice immunized with *L. casei*-expressing intimin fragments (72% and 52% of bacteria with a fluorescence intensity higher than 10^1 for *L. casei*-Int$_{cv}$ and *L. casei*-Int$_{v}$ sera, respectively). Sera obtained from mice immunized with *L. casei* produced a fluorescence intensity similar to sera from the saline group (around 30% of bacteria with a fluorescence intensity higher than 10^1).

Immunization with *L. casei*-Int$_{cv}$ and *L. casei*-Int$_{v}$ induces antibodies that inhibit *in vitro* EPEC O111ab:H2 binding to epithelial cells

To evaluate the functionality of the antibodies induced by immunization with *L. casei*-Int$_{cv}$ and *L. casei*-Int$_{v}$, we first performed an *in vitro* assay commonly used for the determination of EPEC binding to epithelial cells. In this assay, the different sera were incubated previously with EPEC O111ab:H2 to allow antibody binding and the samples were then added to HEp-2 monolayers cultivated on coverslips. EPEC O111ab:H2 displays a localized pattern of adhesion, generating tight microcolonies of bacteria on the epithelial cells surface (supporting Fig. S1). Adhesion was carried out in 4 h and the percentages of cells harboring EPEC

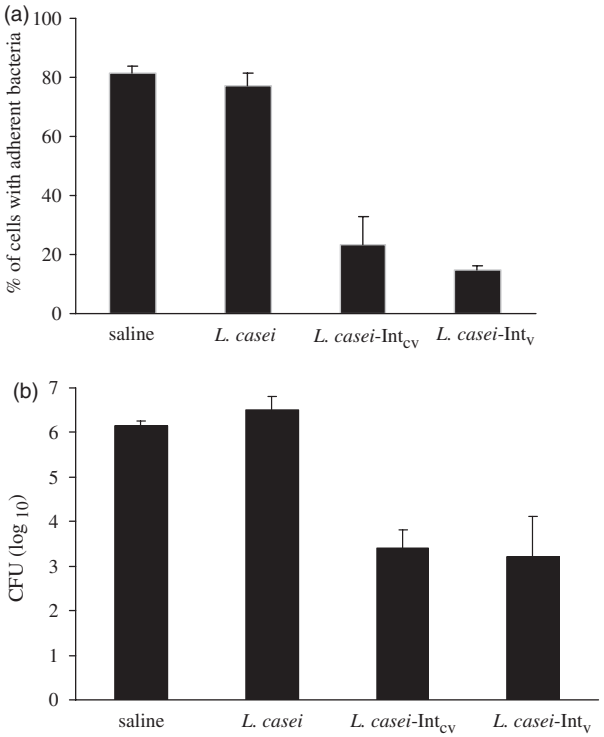

Fig. 4. Inhibition of EPEC O111ab:H2 adhesion to HEp-2 cells. (a) Different sera were incubated with 10^6 bacteria for 1 h. Samples were added to HEp-2 cells cultivated on glass coverslips, incubated for 4 h at 37 °C, washed, fixed and stained. Percentages of cells containing adhered bacteria were calculated after counting with the help of a microscope. Results are expressed as means of percentages with SDs of quadruplicates. (b) Different sera were incubated with 10^6 bacteria for 1 h. Samples were added to HEp-2 cells that were lysed after incubating and washing steps. Recovered bacteria were counted in LB plates. Results are expressed as means of bacteria counting with SDs of quadruplicates and are representative of experiments using sera from two independent immunization experiments.

microcolonies were calculated in stained slides after extensive washings. As shown in Fig. 4a and supporting Fig. S1, 75% and 85% inhibition of EPEC adhesion were observed when bacteria were incubated previously with sera obtained from immunization of mice with *L. casei*-Int$_{cv}$ and *L. casei*-Int$_{v}$, respectively. Both sera from mice inoculated with saline or *L. casei* revealed an inhibition capacity of around 20%, correlating with the results observed in FACS analysis.

Quantification of inhibition of EPEC adhesion was also analyzed through the lysis of HEp-2 cells after the binding assay and plating the suspension on LB for counting of the total bacteria adhered. Using this method, we were able to determine decreases of around 2 logs in bacteria recovery by sera from mice immunized with *L. casei*-Int$_{cv}$ and *L. casei*-Int$_{v}$, confirming the results obtained by counting of microcolonies (Fig. 4b). Similar amounts of bacteria were recovered when the sera from mice inoculated with saline or *L. casei* were used.

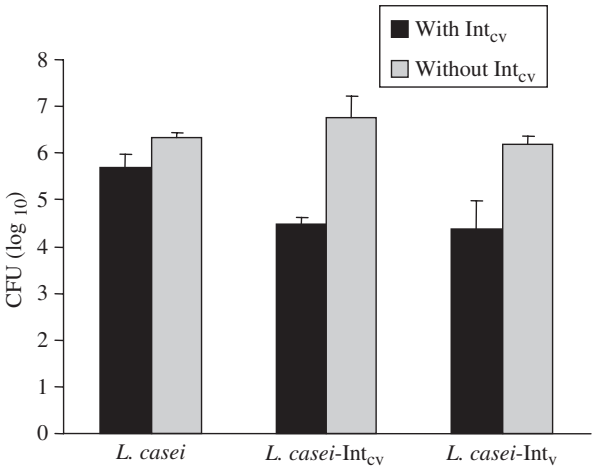

Fig. 5. Reversion of EPEC adhesion inhibition by recombinant Int$_{cv}$. Different sera were incubated with 4 μg of *Escherichia coli*-produced recombinant Int$_{cv}$ before incubation with EPEC O111ab:H2. Samples were added to HEp-2 cells, which were lysed after a 4-h incubation. Recovered bacteria were counted in LB plates. Results are expressed as means of bacteria counting with SDs of quadruplicates.

In order to determine whether the inhibition of adhesion observed was really due to antibody binding to intimin, sera were incubated previously with purified Int$_{cv}$ before performing the adhesion assay. The results showed that the inhibition exerted by both sera was completely abolished by the previous incubation of sera with the recombinant protein (Fig. 5).

Immunization with *L. casei*-Int$_{cv}$ and *L. casei*-Int$_{v}$ induces antibodies that inhibit *in vitro* EPEC E2348/69 binding to epithelial cells

Because the Int$_{cv}$ fragment includes a conserved region that displays 83% similarity to the amino acid sequence of intimin α from EPEC E2348/69, we decided to analyze the effect of the different sera on the *in vitro* binding of this strain to epithelial cells. As can be observed in Fig. 6, incubation of the E2348/69 strain with sera from mice immunized with *L. casei*-Int$_{cv}$ inhibited bacteria adhesion to a level similar to that observed for the homologous O111ab:H2 strain. A less pronounced inhibition was elicited by sera from mice immunized with the *L. casei*-Int$_{v}$. This result correlates with a lower amino acid similarity between the two intimins in this region (61% similarity). No inhibitory effect was observed with sera from mice inoculated with saline or *L. casei*.

Discussion

Intimin is an essential EPEC virulence factor (Frankel *et al.*, 1996; Cleary *et al.*, 2004; Miyake *et al.*, 2005), also common to other human diarrhea-causing bacteria such as enterohemorragic *E. coli* (EHEC) and animal pathogens such as

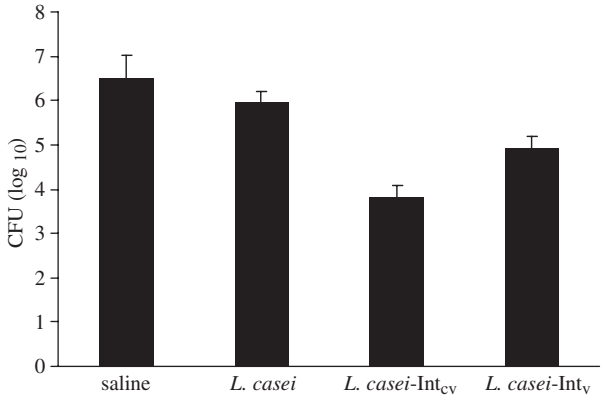

Fig. 6. Inhibition of EPEC E2348/69 adhesion to HEp-2 cells. Different sera were incubated with 10^6 bacteria for 1 h. Samples were added to HEp-2 cells that were lysed after a 3-h incubation. Recovered bacteria were counted in LB plates. Results are expressed as means of bacteria counting with SDs of quadruplicates and are representative of experiments using sera from two independent immunization experiments.

Citrobacter rodentium and rabbit diarrheagenic *E. coli* (REPEC). Antibodies against this protein are induced after infection of both humans and animals and are related to protection and inhibition of bacterial adhesion to epithelial cells *in vitro* (Cravioto *et al.*, 1991; Camara *et al.*, 1994; Loureiro *et al.*, 1998; Gansheroff *et al.*, 1999; Carbonare *et al.*, 2003). Studies on the structure and antigenic variability of intimin have determined that the region between 1 and 660 aa comprises a conserved sequence of around 80% similarity among intimin molecules. On the other hand, the region that corresponds to the last 280 residues (Int_{280}) is characterized by its variability (around 40% of amino acid similarity among intimin molecules) that leads to the classification of intimins into different subtypes (Tarr & Whittam, 2002; Zhang *et al.*, 2002; Ito *et al.*, 2007).

In the present study, two different fragments of intimin β were constitutively expressed in *L. casei*. Nasal immunization of mice with recombinant bacteria induced specific antibodies in sera (IgG) and nasal washes (sIgA). Surprisingly, a consistent reactivity was also observed in sera and nasal wash samples from mice immunized with *L. casei* carrying the empty vector as well as nonrecombinant *L. casei*. Analysis on the similarity between intimin from EPEC O111ab:H2 and *L. casei* amino acid sequences deposited in GenBank did not indicate any significant result. Nevertheless, the complete genome of the *L. casei* CECT5275 is not available and a possible similarity among protein primary structures cannot be excluded completely. Further investigation on the reactivity of the sera collected from immunized mice indicated a specific recognition of intimin in EPEC protein extracts. Although both sera from mice immunized with *L. casei*-Int_{cv} and *L. casei* do react with intimin, reactivity displayed by the sera from the latter group seems to be lower (Fig. 3a). Immunization of mice with *L. casei* also induces antibodies against *L. casei* proteins (data not shown), confirming the presence of immunogenic proteins in the live vector used. Despite the immunoreactivity of sera from control *L. casei* with intimin β observed in ELISA and immunoblot, only the sera from mice immunized with *L. casei*-Int_{cv} and *L. casei*-Int_{v} were able to bind to the surface of EPEC O111ab:H2. One explanation for these results is that the reactivity of antibodies induced by immunization with *L. casei* is directed towards a region of intimin that is not exposed in the EPEC surface, because the N-terminal part of the Int_{cv} fragment is predicted to be inside the bacterial extracellular membrane (Luo *et al.*, 2000). Alternatively, the site(s) of recognition by these antibodies may be hidden by the folding of intimin while attached to the EPEC surface.

Int_{280} can be divided into two immunoglobulin-like domains (D1 and D2) and one lectin-like domain (D3) (Frankel *et al.*, 1995; Luo *et al.*, 2000). Despite the variability of this region, some amino acid residues such as W_{899}, C_{860} and C_{937}, located in D3, are highly conserved and were shown to be important for protein stability, binding to the receptor Tir and/or induction of A/E lesions (Batchelor *et al.*, 2000; Luo *et al.*, 2000). A minimal intimin fragment able to bind Tir is composed of the last 190 amino acids in the Int_{280} sequence, although with apparently less affinity than the entire Int_{280} (Batchelor *et al.*, 2000). Despite the localization of the Tir-binding site at the C-terminal end of Int_{280}, analysis of the immunodominant regions inside Int_{280} has shown that antibodies against this fragment are directed towards two regions in the N-terminal of Int_{280} more precisely, a region located in the first 80 aa and a region between aa 80 and 130 of Int_{280} (Adu-Bobie *et al.*, 1998). Most importantly, human colostrum samples specifically recognize these fragments but not the C-terminal region of Int_{280} (Adu-Bobie *et al.*, 1998). Thus, natural exposure to intimin-bearing pathogens seems to elicit antibodies that are reactive only to the N-terminal region of Int_{280}. In the present work, the intimin fragments expressed in *L. casei* include the first 147 aa residues of Int_{280} and therefore the immunodominant region. Sera from mice immunized with *L. casei*-Int_{cv} or *L. casei*-Int_{v} were able to inhibit EPEC adhesion to epithelial cells *in vitro*, in contrast with sera from the group immunized with the control *L. casei*. Thus, our results confirm that sera raised against the first region of Int_{280} are able to inhibit EPEC adhesion to epithelial cells. A large body of evidence indicates that besides Tir, intimin also interacts with host cell-encoded receptors (Sinclair *et al.*, 2006). Still, the relevance of these interactions *in vivo* and their molecular characterizations are not completely understood (Frankel *et al.*, 2001). It is possible that the antibodies induced against Int_{cv} and Int_{v} also block these interactions.

Additionally, sera from mice immunized with *L. casei* expressing the Int_{cv} fragment, which displays 83% similarity to amino acids from the corresponding region of intimin α from EPEC E2348/69, were also able to inhibit adhesion of this strain to HEp-2 cells. Accordingly, a less pronounced inhibition of EPEC E2348/69 adhesion was observed when sera from mice immunized with *L. casei*-expressing Int_v, which displays 61% similarity to amino acids from the corresponding region of E2348/69 intimin, were used. This significant result suggests that the intimin Int_{cv} fragment may be a good candidate for the composition of a vaccine formulation with a broader coverage among *E. coli* expressing different intimin serotypes.

The beneficial effect of probiotics against enteropathogens has been described extensively (Huang *et al.*, 2002; Canani *et al.*, 2007; Pant *et al.*, 2007). Using an *in vitro* assay, Sherman *et al.* (2005) were able to demonstrate that two *Lactobacillus* strains prevent EPEC and EHEC adhesion and consequent injuries to T84 epithelial cells. In addition to a plausible competition to the site of infection, these bacteria also activate the innate immune system (Mohamadzadeh *et al.*, 2005; Galdeano & Perdigon, 2006), aiding in the combat against enteropathogens. In this context, secreted peptides, such as the one present in *Lactobacillus helveticus*-fermented milk, were also shown to modulate immune responses against EHEC infection in mice (Leblanc *et al.*, 2004). However, all these benefits will depend on regular consumption of these bacteria. In the present work, we show that intimin presentation by *L. casei* may provide an additional line of protection that is conferred by the induction of antibodies.

In addition to the induction of systemic antibodies, nasal immunization with our recombinant bacteria also induced specific anti-intimin sIgA in nasal washes. We have tested these samples for inhibitory activity of EPEC adhesion to HEp-2 cells, but none of them have shown this capacity (data not shown). It is possible that the levels of sIgA are too low for the *in vitro* assay used in our studies.

Oral administration of *L. casei* expressing the flagellar antigen from *Salmonella enterica* serovar Enteritidis induced protective cellular immune responses, but low levels of humoral responses (Kajikawa *et al.*, 2007). The authors report that the levels of antigen produced by the bacteria may not be sufficient to induce mucosal immune responses. In accordance with these results, we were not able to detect significant levels of anti-intimin antibodies in saliva or feces samples in mice immunized with *L. casei*-Int_{cv} or *L. casei*-Int_v through the oral route (data not shown). It is possible that the use of additional mucosal adjuvants is necessary for the induction of humoral responses at the intestinal level. Another possibility includes the combination of immunization routes in prime-boost experiments. Oral immunization of mice with recombinant *Lactobacillus reuteri* expressing a chimera of the heat-stable and heat-labile enterotoxigenic *E. coli* enterotoxin ($STLT_B$) induced specific anti-$STLT_B$ antibodies and neutralization of enterotoxin in a mouse model (Wu & Chung, 2007). Thus, the use of other *Lactobacillus* species for the expression of intimin may also be an interesting approach. Effective and affordable vaccines against infantile diarrhea are critical for a reduction in mortality among children and improvement of life conditions in developing countries. The present work moves towards this purpose, showing that immunization of mice with *L. casei* expressing different intimin fragments elicits antibodies that are able to bind to the EPEC surface and inhibit its adhesion to epithelial cells.

Acknowledgements

We are very grateful to Debora Andrade Silva and Felipe Macedo for technical assistance, Dr Jorge M.C. Ferreira Jr for help with FACS analysis and Vânia Gomes de M. Mattaria for the animal facilities' coordination. This work was supported by CNPq, FAPESP and Fundação Butantan.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Inhibition of EPEC O111ab:H2 adhesion to HEp-2 cells.

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