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Expression of the *Lactobacillus Plantarum* Surface Layer MIMP Protein Protected NCM460 Epithelial Cells from Enteroinvasive *Escherichia Coli* Infection

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Key Words

Micro integral membrane protein • *Lactobacillus plantarum* • NCM460 • Occludin • Protein kinase C-η • Probiotics

Abstract

Our previous studies demonstrated that the micro integral membrane protein (MIMP) located within the integral membrane protein of *Lactobacillus plantarum* CGMCC 1258 protected the intestinal barrier from injury. To further analyze the protective effects conferred to intestinal epithelial cells by MIMP, we established transient MIMP-expressing NCM460 cells (NCM460/MIMP) as a means of assessing their susceptibility to infection. We constructed a recombinant eukaryotic expression vector using pcDNA3.1(-) and the MIMP gene. The recombinant vector was then transduced into NCM460 cells and the anti-infective properties of the transient MIMP-expressing NCM460/MIMP cells assessed. Flow cytometric analysis revealed that 82.16%±12.22% of NCM460/MIMP cells expressed MIMP and Western blot analysis confirmed high levels of MIMP expression. Attachment assays showed that the ability of enteropathogenic *Escherichia coli* (EPEC) to attach to NCM460/MIMP cells decreased significantly compared to adhesion observed to NCM460 cells. Western blot and quantitative RT-PCR showed that the expression levels of tight junction (TJ) proteins, including claudin-1, occludin, JAM-1 and ZO-1 in NCM460/MIMP cells infected with EPEC were similar to levels observed in uninfected NCM460 cells. Fluorescence further showed that NCM460/MIMP cells had significantly higher TJ protein staining intensity ocompared to NCM460 cells and transmission electron microscopy indicated that TJ structure was unchanged in NCM460/MIMP cells infected with EPEC compared to NCM460 cells after EPEC infection. Expression levels of PKC-η and phosphorylated occludin were also higher after EPEC infection in NCM460/MIMP cells compared to expression levels in EPEC-infected NCM460 cells. These data demonstrated that NCM460/MIMP cells possessed EPEC anti-infective properties related to the activation of protein kinase C-η and occludin phosphorylation.

Introduction

Maintenance of gut flora homeostasis in the human intestine is mediated largely in part by bacteria of the

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genus lactobacilli and other probiotic organisms [1-2]. It has been demonstrated that probiotic (with organisms such as lactobacilli) treatment of patients presenting with colonic infections or inflammatory disorders over the past decades has been beneficial [3-5] due to the ability of lactobacilli to mediate gut flora homeostasis, improve intestinal epithelial barrier function and modulate epithelial and immune cell function [6]. Furthermore, the interaction between the lactobacillus surface layer protein (SLP) and intestinal epithelial cells has been recognized as a key component mediating the protection conferred by lactobacillus to human intestinal epithelial cells [7-8].

Lactobacilli adhesion to human intestinal tract epithelial cells is a critical first step involved in the regulation of intestinal cell function. Attachment is initiated by non-specific physical interactions between the surface of lactobacilli and human intestinal epithelial cells. This interaction is followed by the activation of yet to be defined lactobacillus ligands binding to their corresponding receptors on intestinal epithelial cells [9-10]. Ligands on the surface of the lactobacilli are adhesive proteins that can modulate gut-barrier function and local systemic inflammatory responses by mediating lactobacillus adhesion to the target intestinal tract cells and subsequently activating corresponding signal transduction pathways [11-12]. These adhesive proteins (also referred to as adhesins) may also competitively block the adhesion of pathogenic bacteria such as enteroinvasive *E. coli* (EIEC) and enteropathogenic *E. coli* (EPEC) to the epithelial cell surface [12-13].

Lactobacillus SLPs have been shown to be surface structures critical to the interaction between lactobacilli and human intestinal epithelial cells. Although some of the structural regions of the SLPs may be responsible for lactobacillus adhesion to the intestinal epithelium, only a few studies have explored the role of SLPs in mediating epithelial attachment and fewer studies have attempted to define SLP binding domains due to unique SLP hydrophilic and hydrophobic properties and technical difficulties associated with SLP purification [8, 13-14]. The normal human colon-derived mucosal epithelial cell line NCM460 (which expresses colonic epithelial cell-associated antigens such as cytokeratins and villin) has been widely used in various studies involving the interactions between the intestinal epithelium and infectious agents [15-17].

Our previous studies indicated that *L. plantarum* (LP) conferred therapeutic effects by adhering to epithelial cells, restoring tight junction (TJ) structure and function and improving paracellular permeability both *in vivo* and *in vitro* [18-23]. Furthermore, our recent study (manuscript in press) describing SLP functional domains indicated that the LP strain CGMCC 1258 integrated membrane protein (IMP) mediated adherance to intestinal epithelial cells (IECs). We further determined that IMP-2 was responsible IMP adhesion to human IECs. Moreover, micro IMP (MIMP, IMP515-575), the small active domain adhesive protein within IMP-2, was further purified and successfully characterized using bioinformatics and molecular techniques. Competitive inhibition assays were performed in this study to further confirm the ability of MIMP to interfere with EPEC adherence to NCM460. Our previous data (manuscript in press) also confirmed that MIMP reduced intestinal permeability and restored the expression and distribution of TJ proteins in both NCM460 cell monolayers and in IL-$10^{-/-}$ mice. MIMP also adhered to immature DCs by binding to DC-SIGN, inducing DCs to produce anti-inflammatory cytokines and to mediate Th2 differentiation. Moreover, MIMP stimulated the expression of anti-inflammatory cytokines in colonic mucosa and attenuated colitis in IL-$10^{-/-}$ mice (manuscript in press). Therefore, our above mentioned findings indicated that MIMP was the main LP component that conferred protective effects on IECs, establishing a foundation from which the anti-infective role of MIMP could be further defined. The aim of our study was to establish and characterize the anti-infective properties of NCM460/MIMP, cells that transiently expressed MIMP (the SLP binding domain of LP) to further elucidate the protective effects of MIMP on IECs against bacterial infection.

Materials and Methods

Bacterial strains and culture conditions

The EPEC strain ATCC 43887 (O111:NM) (Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China) was grown in DMEM at 37°C for 24 h. Quantification of bacteria was carried out by measuring the optical density at 600 nm (Beckman DU-50 spectrophotometer) to determine the colony forming units (cfu).

Culture of NCM460 cells

NCM460 cells were purchased from INCELL Corporation (San Antonio, TX, USA) and cultured in M3 media supplemented with 10% FBS (fetal bovine serum), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 95% humidified atmosphere with 5% CO2. Cells were passaged at pre-confluent densities using 0.05 % trypsin and 0.5 mm EDTA (Invitrogen,

Carlsbad, CA) [24]. NCM460 cells were passaged 24 h before transfection.

Transfection of NCM460 cells with MIMP and construction of NCM460/MIMP cells

PCR was carried out in a total volume in 100 µL containing 5 µL genomic DNA, 20 pmol of each primer, 20 µmol dNTPs, 1X *Pyrococcus furiosus* (*pfu*) reaction buffer and 5 U *pfu* DNA polymerase. PCR was performed under the following conditions: genomic DNA was denatured at 94°C for 3 min followed by 30 amplification cycles (94°C for 30 s, 56°C for 30 s, and 72°C for 2 min) followed by a final extension at 72°C for 15 min. The MIMP gene was PCR amplified from genomic DNA using the following corresponding primer pairs: Forward primer, 5'-GAA TTC ACT CAC ACG GTT GGA-3' and reverse primer, 5'-CTC GAG TTG TTT CTT CAA CTG GT-3'. PCR products were identified by subjecting the reaction mixtures to 3% gel electrophoresis. The PCR products were recovered with the DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). After digestion with *Eco*RI and *Xho*I (MBI Fermentas, Glen Burnie Maryland, USA) PCR products were inserted into eukaryotic expression vector pcDNA3.1(-) (Invitrogen) and ligated using T4 DNA ligase. Recombinant expression plasmid and lipofecter (Lipofectamine™ 2000, Invitrogen) were mixed at a ratio of 1:1-2:1 (lipofecter:MIMP DNA) in 100 µl M3 serum free media by gently swirling or inverting the container for 25 min. NCM460 cell supernatants were removed and cells washed 3 times with M3 serum free media and cultured with the PCR products and lipofecter for 1 h at 37°C in a 95% humidified atmosphere with 5% CO_2. The medium was then replaced with M3 media and MIMP expression confirmed using polyclonal anti-MIMP antibodies (manuscript in press).

Fluorescence-activated cell sorting (FACS)

Flow cytometry was performed as previously described [25-27]. Anti-MIMP antibodies were prepared by immunizing an adult male New Zealand white rabbit with MIMP that was used as the primary antibody diluted 1:600 in PBS-T (phosphate buffered saline-0.1% Tween 20) buffer. The number of NCM460MIMP cells was determined using a fluorescence-labeled anti-rabbit secondary antibody (diluted 1:100 in PBS-T buffer, Abcam, Cambridge, MA, USA). Cells were cultured with anti-MIMP for 2 h, washed and then incubated with the secondary antibody for 45 min. The transfection efficiency was calculated by determining the percentage of cells expressing MIMP on the surface.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis

To confirm whether MIMP was present in transfected cells, protein samples were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [28-29]. Transfected cells were homogenized in chilled RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA) containing protease and phosphotase inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and 5 g/ml of each of aprotinin, leupeptin, pepstatin). SDS-PAGE was performed according to standard laboratory techniques with a discontinuous gradient, 5% (w/v) stacking gel and a 10% (w/v) separating gel, in a Miniprotean II (Bio-Rad). Briefly, samples were mixed with loading buffer containing SDS and mercaptoethanol, boiled for 3 min, centrifuged and subjected to SDS-PAGE. Gels were then transferred onto PVDF membranes (Millipore, MA, USA) using a semidry electroblotter (Bio-Rad) for 120 min at 100 V. Membranes were washed three times (20 min each) with PBS-T buffer. After blocking overnight in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% powdered milk, membranes were washed 3 times (5 min each) with TBS-T and incubated with polyclonal anti-MIMP antibodies for 2 h at room temperature. After 3 washes with TBS-T, membranes were incubated for 1 h with a goat anti-rabbit secondary antibody (Bio-Rad). The membrane was washed three times (60 min each) with PBS-T buffer and binding visualized using enhanced chemiluminescence (ECL kit; Pierce, IL, USA) according to the manufacturer's instructions.

EPEC binding assay

NCM460 cells grown on M3 base media at 37°C in a 95% humid atmosphere (5% CO_2) for 72 h were washed three times in Hank's balanced solution (Life Technologies, San Diego, CA, USA) to remove the antibiotics. Cells were then cultured as monolayers (~$1x10^7$ for each monolayer) and divided into four different experimental groups in triplicate. NCM460 monolayers cultured without EPEC were used as controls. The EPEC group consisted of 100 µL EPEC at $1.0x10^8$/mL added to the apical side of the cell culture insert for rapid infection of the monolayer. The EPEC:NCM460 cells were inoculated at 100:1 ratio. The insert was placed in a 50 mL tube and centrifuged (200xg for 4 min). The NCM460/MIMP group consisted of the NCM460/MIMP cell monolayer only and for the NCM460/MIMP+EPEC group the cells were treated with 100 µL EPEC at $1.0x10^8$/mL added to the apical side of the NCM460/MIMP cell monolayer.

After incubation of NCM460 or NCM460/MIMP cells for 24 h, cells were trypsinized and the number of adhered EPEC determined by plating diluted bacterial suspensions on MRS or blood agar plates and determining the cfu by counting colonies 24 h later. Cells were counted using a Sysmex SE900 automatic cell counter (Toa Medical Electronics, Kobe, Japan). The adhesion rate (%) = (the number of EPEC adhering to the cells/the overall number of bacteria) x 100%.

Determination of TJ protein expression levels

NCM460/MIMP cells were treated as described above, subjected SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated with the corresponding primary antibodies *i.e.*, antibodies against TJ proteins including anti-claudin-1, anti-occludin, anti-JAM-1 and anti-ZO-1 at a dilution of 1:50 (Abcam) for 2 h at room temperature, washed and then incubated for 1 h with corresponding HRP-conjugated secondary antibodies. The TJ proteins were visualized using enhanced chemiluminescence as above.

Detection of mRNA expression levels of TJ proteins by quantitative real time PCR

TJ protein mRNA expression levels (claudin-1, occludin, JAM-1 and ZO-1) were determined by quantitative real time PCR (qRT-PCR) [30-31]. Primers used in our study included: occludin forward primer, GCA GCT ACT GGA CTC TAC G and reverse primer, ATG GGA CTG TCA ACT CTT TC; claudin forward primer, GTG CCT TGA TGG TGG TTG and reverse primer, TGT TGG GTA AGA GGT TGT; JAM-1 forward primer, GAT GTG CCT GTG GTG CTG and reverse primer GCT CTG CCT TGA GAT AAG AA; ZO-1 forward primer AAG AGT GAA CCA CGA GAC and reverse primer TCC GTG CTA TAC ATT GAG. Total RNA was then isolated from NCM460/MIMP cells using Trizol reagent (Invitrogen) followed by DNase I treatment. The quantity and quality of RNA was verified by determining the absorbance ratio at 260 and 280 nm and by visualization of respective bands on agarose gels. For each sample, 600 ng mRNA was used in the reverse transcription reaction (iScript kit, BioRad) according to the manufacturer's specifications. Further analysis of mRNA levels of each group was performed by RT-PCR using a light-cycling system (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany). The level of mRNA expression was expressed as the ratio of the mean reading of the experimental group over that of the control group for NCM460/MIMP cells.

TJ protein expression level characterization

NCM460 and NCM460/MIMP cells were cultured and monolayers treated as described above, fixed with acetone/methanol (1:1) at 0°C for 5 min, permeabilized in 0.2% Triton X-100 and incubated with primary antibodies (anti-claudin-1, anti-occludin, anti-JAM-1 or anti-ZO-1) followed by an incubation with respective secondary antibodies (fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, Sigma, St. Louis, MO, USA) in 3% nonfat milk as described [18]. Fluorescence was visualized using confocal laser scanning microscopy (MRC 1024, Bio-Rad).

Transmission electron microscopy analysis of TJ protein ultra-structures

NCM460 cells and NCM460/MIMP cells ($1x10^6$ cfu) growing on intact Millicell™ inserts were rinsed with Dulbecco's PBS (D-PBS) and fixed with freshly prepared 2.5% glutaraldehyde, 1.8% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated using a graded ethanol and propylene oxide series and embedded in Epon (Shell Chemical Co., Houston, TX) as previously described [18]. Thin sections were obtained using an ultramicrotome (Reichert-Jung Ltd, Wetzlar, Germany) and stained with uranyl acetate and lead citrate prior to visualization under an FEI TECNAI G2F20 electron microscope (FEI Ltd, Hillsboro, OR).

Detection of PKC-η expression levels by Western blot

Occludin phosphorylation levels were determined in NCM460/MIMP cells not infected with EPEC (control group), NCM460/MIMP cells infected with EPEC (EPEC group) and NCM460/MIMP cells pretreated with a PKC-η inhibitor for 2 h before infection with EPEC (inhibitor group). Expression levels of PKC-η, occludin and phosphorylated occludin (P-occludin) were determined by Western blot as described above. The PKC pseudo substrate peptide [32] Myr-TRKRQRAMRRRVHQING was used as the PKC-η inhibitor and the control peptide with a scrambled sequence (Myr-RMINKARVRGRAQRHG-OH) were custom synthesized by the Institute of Bio-medicine, Shanghai Jiao Da Onlly Company Ltd, Shanghai, China. Primary antibodies to PKC-η, occludin and P-occludin were purchased from Abcam.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD) when normally distributed or as a median (range) when abnormally distributed. Statistical analyses were completed using the SPSS 11.0 system (SPSS Inc., Chicago, IL). SD between multiple groups were assumed to satisfy a normal distribution. Data were analyzed by one-way ANOVA when conditions of homogeneity of variance were present. P values <0.05 were considered to be statistically significant.

Results

NCM460/MIMP cells had high MIMP expression levels

Before challenging NCM460/MIMP cells with EPEC we first determined the MIMP transfection efficiency and surface expression by FACS. FACS analysis demonstrated that the percentage of NCM460/MIMP cells expressing MIMP was 82.16%±12.22%. In addition, MIMP expression levels from NCM460/MIMP cell membrane fractions were also characterized by Western blot and densitometry analysis. The density of MIMP in NCM460/MIMP cells was greater than observed in NCM460 cells and semi-quantitative analysis of Western blots showed that the NCM460/MIMP MIMP expression levels were higher when compared to expression levels of NCM460 cells (Fig. 1, $P<0.05$).

Reduced attachment of EPEC to NCM460/MIMP cells

Attachment of EPEC to NCM460 and NCM460/MIMP was assessed as a means determining if MIMP played a role in interfering with bacterial attachment to human epithelial cells. This analysis demonstrated that EPEC had an adhesion rate of 38.8% to NCM460 cells compared to a significantly reduced attachment rate of 20.8% to NCM460/MIMP, demonstrating the anti-infective phenotype conferred by MIMP (Fig. 2, $P<0.05$).

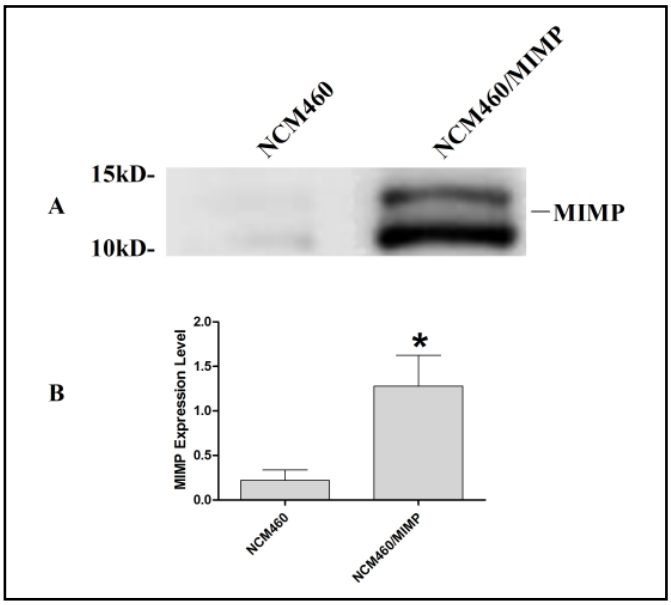


Fig. 1. MIMP expression by NCM460/MIMP cells. (A) Western blot analysis of MIMP expression by NCM460/MIMP and NCM460 cells. (B) Semi-quantitative densitometric analysis of the Western blot shown in (A). *P<0.05 vs. EPEC treated cells. One-way ANOVA was performed with Tukey-Kramer post-hoc comparison. Values were calculated using the Student's *t*-test. All data are expressed as the mean±SE.

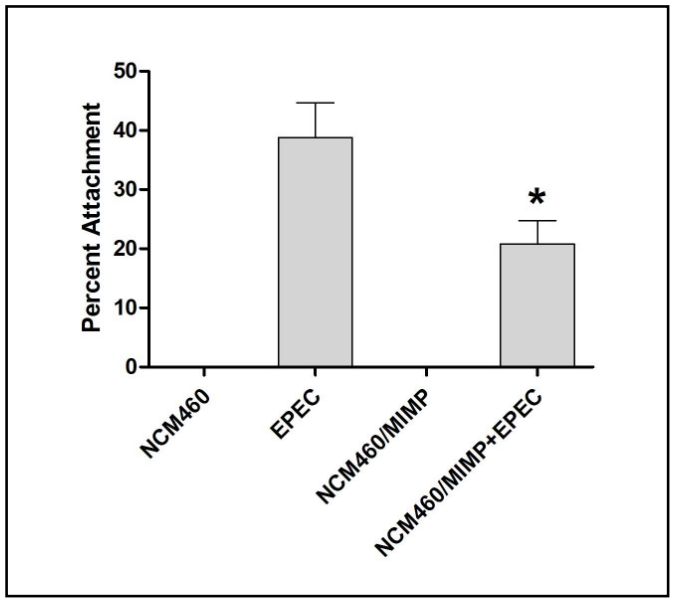
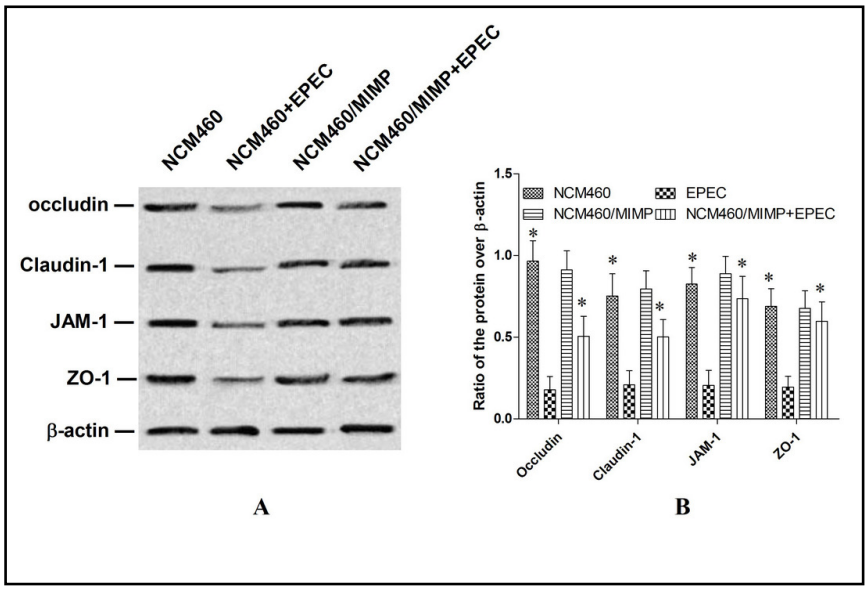


Fig. 2. Adhesion of EPEC to NCM460/MIMP cells. EPEC adhesion to NCM460/MIMP was assessed after 24 h of culture. $1.0x10^8$/mL EPEC were added to respective cells and quantified by determining cfus. *P<0.05 vs. EPEC group. One-way ANOVA was performed with Tukey-Kramer post-hoc comparison. Values were calculated by Student's *t*-test. P < 0.05

Fig. 3. TJ protein expression following EPEC infection. (A) NCM460/MIMP or NCM460 cells were incubated in the presence or absence of EPEC and whole cell lysates analyzed for the presence of TJ proteins (claudin-1, occludin, JAM-1 and ZO-1) by Western blot analysis. (B) Semi-quantitative analysis of Western blots showed that the expression levels of claudin-1, occludin, JAM-1 and ZO-1 were decreased in NCM460 cells infected with EPEC compared to the control group. However, in NCM460/MIMP cells the expression levels of tight junction proteins was elevated compared to NCM460 cells. *P<0.05 vs. EPEC group. One-way ANOVA was performed using Tukey-Kramer post-hoc comparison. Values were calculated using the Student's *t*-test. All data are expressed as the mean±SE.



NCM460/MIMP TJ proteins were protected from EPEC-mediated cellular changes

TJ protein expression levels *i.e.*, claudin-1, occludin, JAM-1 and ZO-1, were analyzed in membrane cellular fractions by immunoblotting and subsequent densitometric analysis. Western blot analysis of epithelial whole-cell protein extracts showed that TJ protein expression was higher in NCM460/MIMP than in NCM460 cells after infection with EPEC (Fig. 3A). Semi-quantitative analysis of NCM460/MIMP lysates by Western blot showed that the expression levels of claudin-1, occludin, JAM-1 and ZO-1 were decreased in NCM460 cells infected with

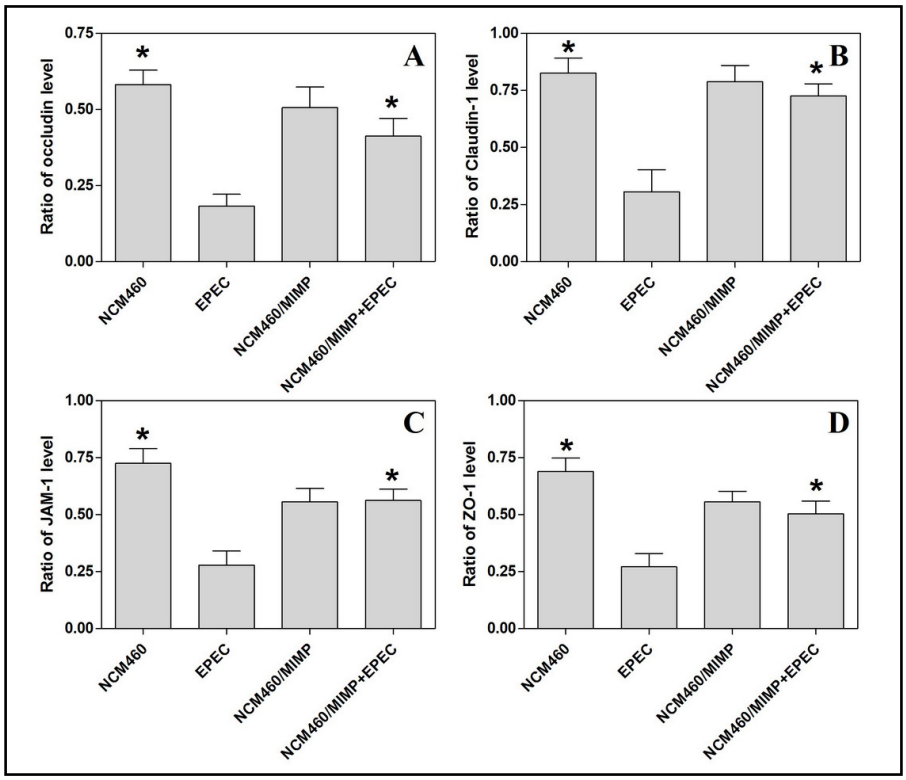


Fig. 4. NCM460/MIMP cells were protected against EPEC-mediated changes to TJ protein mRNA. TJ protein mRNA levels were assessed in NCM460/MIMP or NCM460 cells in the absence or presence of EPEC. Quantitative RT-PCR analysis of the expression ratio of (A) occluding, (B) claudin-1, (C) JAM-1 and (D) ZO-1 in the different treatment groups showed that mRNA levels of TJ proteins was higher in NCM460/MIMP cells infected with EPEC compared to the NCM460 cells. *P<0.05 vs. EPEC group. One-way ANOVA was performed with Tukey-Kramer post-hoc comparison. Values were calculated using the Student's t-test. All data are expressed as the mean±SE.

EPEC compared to the control group. However, TJ protein expression levels in NCM460/MIMP cells were elevated significantly compared to levels observed in NCM460 cells incubated with EPEC (Fig. 3B, P<0.05).

NCM460/MIMP cells were protected from EPEC-mediated changes to TJ protein mRNA expression

There was a significant decrease in TJ protein mRNA expression (claudin-1, occludin, JAM-1 and ZO-1) in NCM460 cells infected with EPEC compared to the uninfected control. However, NCM460/MIMP cells infected with EPEC had significantly increased levels of TJ protein mRNA compared to NCM460 cells infected with EPEC (Fig. 4, P<0.05).

TJ proteins remained intact in NCM460/MIMP cells

To assess the distribution of TJ proteins in NCM460 and NCM460/MIMP cells cultured in the presence or absence of EPEC, the fluorescence intensity of TJ proteins distributed throughout the cell monolayers was examined. This analysis demonstrated a uniform distribution of TJ proteins in untreated controls, however, EPEC treatment of NCM460 cells resulted in a decrease in TJ protein fluorescence intensity compared to NCM460/MIMP cells cultured with EPEC (Fig. 5).

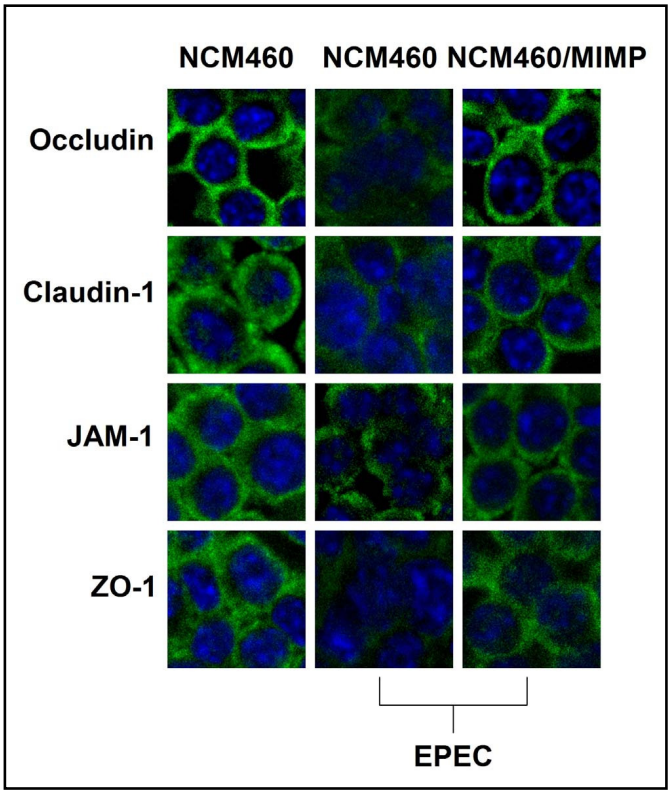


Fig. 5. Fluorescence staining of TJ proteins. Line 1, High fluorescence intensity of TJ proteins in NCM460 cells only; line 2, low fluorescence intensity of TJ proteins in NCM460 cells infected with EPEC; line 3, high fluorescence intensity of TJ proteins in NCM460/MIMP cells infected with EPEC. Magnification 630X.

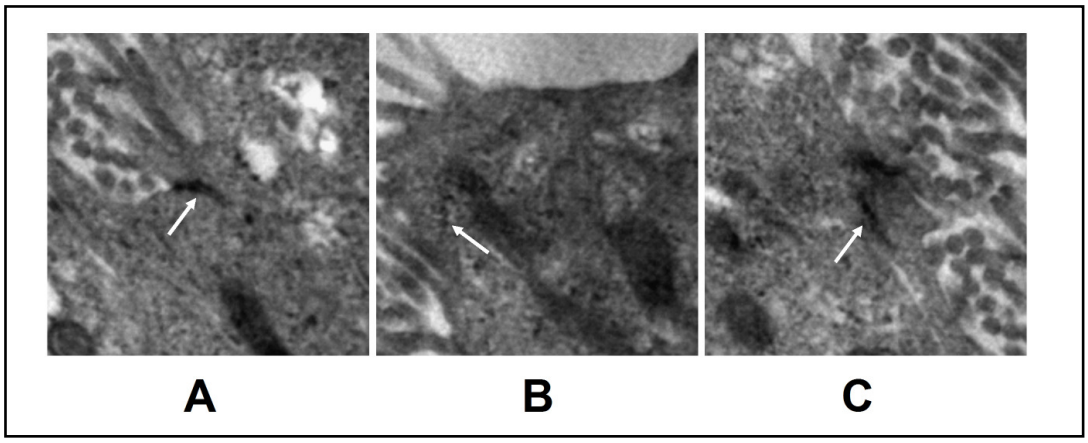


Fig. 6. Transmission electron microscopy analysis. Ultra-structure of TJs from (A) NCM460 cells, (B) NCM460 cells infected with EPEC and (C) NCM460/MIMP cells infected with EPEC. Magnification 97,000X.

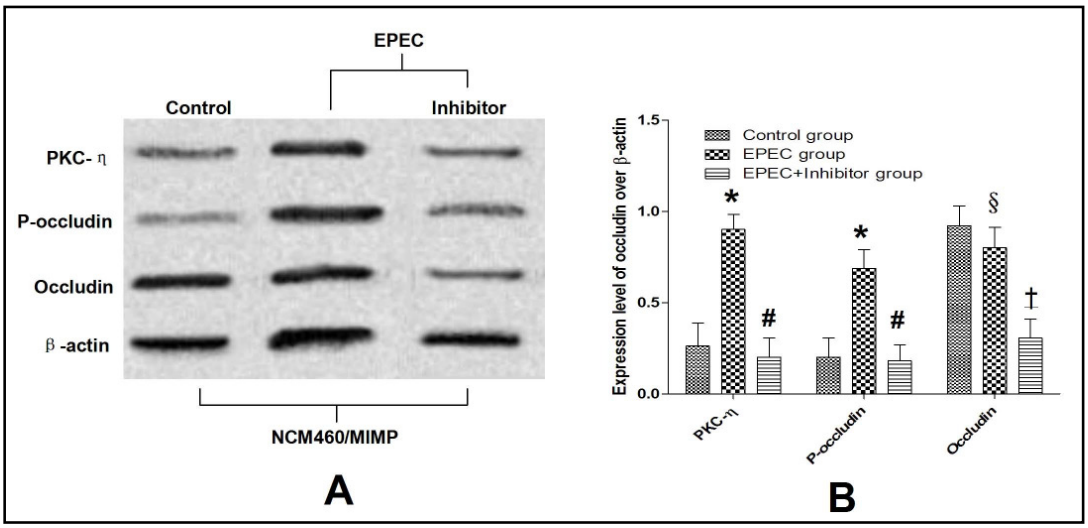


Fig. 7. Expression levels of PKC-η, P-occludin and occludin. Expression levels of PKC-η and P-occludin were assessed by Western blot. (A) Line 1, NCM460/MIMP cells only; line 2, NCM460/MIMP cells infected with EPEC; line 3, NCM460/MIMP cells pre-treated with a PKC-η inhibitor for 2 h prior to EPEC infection. (B) Semi-quantitative analysis of Western blots. *P<0.05 vs. control group; #P<0.05 vs. EPEC group; §P>0.05 vs. control group and † vs. § group, P<0.05. One-way ANOVA was performed with Tukey-Kramer post-hoc comparison. Values were calculated by Student's *t*-test. All data are expressed as the mean±SE.

The TJ structure of NCM460/MIMP cells remained intact after culture with EPEC

The protective effects conferred by MIMP were further analyzed by electron microscopy to assess TJ ultra-structure. Results demonstrated TJs at the top of the membrane between adjacent NCM460 cells in the control group (Fig. 6A). After infection with EPEC, TJ integrity was destroyed and cells presented with irregular microvilli, a marked increase in the intercellular gaps, vacuolization and chromatin condensation compared to uninfected NCM460 cells (Fig. 6B). By contrast, the TJ structure of NCM460/MIMP cultured in the presence of EPEC remained intact (Fig. 6C).

PKC-η-mediated phosphorylation of occluding in NCM460/MIMP cells

Expression levels of PKC-η, P-occludin (phosphorylated occludin) and occludin in NCM460 and NCM460/MIMP cells cultured in the presence or absence of EPEC were determined by Western blot analysis (Fig. 7). After culture with EPEC, NCM460/MIMP cells had significantly elevated PKC-η expression levels compared to the untreated control (P<0.05, Fig. 7B). Expression levels of P-occludin were higher in the NCM460/MIMP cells after infection with EPEC (compared to NCM460 cells infected with EPEC), however, the elevated expression levels were down-

regulated following the addition of a PKC-η inhibitor (Fig. 7B, $P<0.05$). Occludin expression levels were not significantly decreased in the EPEC group compared to the control group (Fig. 7B, $P>0.05$) and pretreatment with a PKC-η inhibitor lead to a lower occluding expression levels (Fig. 7B, $P<0.05$).

Discussion

Probiotics like LP have been reported to have beneficial effects in patients with intestinal infections [33]. As a means of better understanding the biology behind the beneficial effects conferred by LP, our group identified and characterized the MIMP protein and determined that this molecule played a key role in conferring protection against pathogenic bacteria via undefined mechanisms (manuscript in press). To further characterize the protective effects of MIMP on epithelial cells we generated NCM460/MIMP cells, that is, intestinal epithelial cells (NCM460) expressing the IMP domain of the MIMP protein to assess if this MIMP domain conferred protection against infection with EPEC.

To assess the role of MIMP in an *in vitro* infection model we first constructed a recombinant eukaryotic expression vector using the pcDNA 3.1(-) vector and the MIMP gene. The recombinant vectors were then transduced into NCM460 to generate the NCM460/MIMP cells. Before each EPEC challenge experiment, MIMP surface expression was confirmed by FACS as a means of determining transfection efficiency. This analysis demonstrated that 82.16%±12.22% of transduced NCM460 expressed MIMP. MIMP expression levels were characterized by Western blot analysis that demonstrated that NCM460/MIMP cells expressed significantly higher MIMP levels compared to NCM460 cells. This further confirmed the successful transduction and expression of MIMP into NCM460 cells.

The ability of EPEC to adhere to NCM460/MIMP cells was assessed as a means of testing if MIMP served as anti-infective. Results showed that the EPEC adhesion to NCM460/MIMP decreased significantly compared to attachment observed to NCM460 cells, suggesting that MIMP protected NCM460 cells by preventing EPEC attachment. Western blot, quantitative RT-PCR and fluorescence staining were performed to further characterize the protective mechanism conferred by MIMP and demonstrated that in the absence of MIMP the integrity of TJs was compromised. NCM460/MIMP cells had significantly increased expression levels and higher fluorescence intensity of TJ proteins post EPEC infection compared to NCM460 cells infected with EPEC. Transmission electron microscopy demonstrated further that the TJ structure was maintained in NCM460/MIMP cells infected with EPEC compared to NCM460 cells infected with EPEC. Maintenance of TJ integrity in transduced and infected epithelial cells was likely due to MIMP-mediated interference of EPEC attachment which allowed TJ protein (claudin-1, occludin, JAM-1 and ZO-1) expression to remain at normal levels even after infection with EPEC.

Previous studies showed that occludin phosphorylation protected IECs subjected to harmful stimuli (such as bacterial infections) and that PKC-η played an important role in mediating this process [32, 34]. However, no studies had investigated the relationship between infection and IEC PKC-η and P-occludin expression levels. Analysis of PKC-η and P-occludin expression levels in NCM460/MIMP cells was maintained following EPEC infection, maintaining normal occluding expression levels that could be increased if the cells were treated with a PKC-η inhibitor which blocked the anti-infective effects of NCM460/MIMP cells due to reduced occludin expression levels. Data from this study indicated that the protective effects conferred by MIMP might be due to PKC-η activation resulting in occludin phosphorylation which in turn maintained TJ protein integrity and intestinal barrier function [32, 34]. Furthermore, it was reported that phosphorylation of threonine residues (T403 and T404) in PKC-ç in turn regulated occludin phosphorylation essential to the maintenance of intestinal barrier function [32]. However, we did not verify phosphorylatioin of these threonine residues because we did not have the antibodies to carry out that analysis, however, this characterization will be part of future work.

Another limitation of our study was that the protective mechanism conferred by MIMP remains unclear. Studies on barrier function mediated by IECs and their protective mechanisms are drawing more attention and include studies examining intestinal villi brush border alkaline phosphatase (IAP) [35-37], intracyto-plasmic protein phosphatase 2A (PP2A) [38-39], intraepithelial intestinal lymphocytes (IELs) [40-42] and intestinal stem cells (ISC) [43-47] which should be further studied in the context of MIMP. MIMP knock-out LP studies have now been conducted to further investigate the protective mechanism of MIMP and have demonsrated that LP without MIMP had a significantly decreased ability in maintaining intestinal barrier integrity.

Further investigation of the underlying molecular and cellular mechanisms such as cell signal transduction pathways affected by MIMP are in progress.

Conclusion

NCM460/MIMP cells possessed anti-infective properties that related to PKC-η activation resulting in occludin phosphorylation. These changes resulted in a significant ability of EPEC cells to attach to NCM460/MIMP, suggesting that one of the benefits conferred by probiotics such as LP is reducing the attachment of pathogenic bacteria to intestinal epithelial cells.

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