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INTESTINAL MMC-RELATED ELECTRIC FIELDS AND PANCREATIC JUICE CONTROL THE ADHESION OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA TO THE GUT EPITHELIUM - *IN VITRO* STUDY

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The adhesion of six different *Lactobacillus* and *Lactococcus* and three pathogenic *Escherichia* and *Salmonella* strains was studied using Caco-2 cell line. In this *in vitro* model system the influence of weak electric field (EF) on bacterial adhesion was tested. The EF source was the *in vitro* reconstruction of spiking potentials recorded in the duodenum of a healthy calf during one myoelectrical migration complex (MMC) cycle. The ability to adhere to Caco-2 cells of bacteria belonging to two groups, Gram-positive lactobacilli and lactococci, and Gram-negative *Escherichia* and *Salmonella* differed considerably. The pathogenic bacteria adhered better to well-differentiated Caco-2 cells whereas lactobacilli and lactococci displayed better adhesion to non-differentiated Caco-2 cells. In the presence of MMC-related EF an increased adhesion of *Lactobacillus* and *Lactococcus* but not of *Salmonella enterica s.* Enteritidis and *E. coli* 269 to Caco-2 cells was observed. Two later strains adhered even less in the presence of EF. The same tendency was found in the presence of pancreatic juice in a cell medium. In conclusion, the myoelectric component of the small intestinal motility, the MMC-related EF, and pancreatic juice may increase the ability of lactic acid bacteria to adhere to GI epithelial cells, creating better environmental conditions for colonization of the intestine and competition with Gram-negative pathogens.

Key words: *adhesion, Caco-2, bacteria, MMC-related electric field*

INTRODUCTION

Normal gastrointestinal (GI) epithelium is covered with a mucus layer which together with host intestinal microflora is indispensable to protect the epithelial cells against pathogenic bacteria and physical and chemical damage thereby maintaining proper function of the small intestine. The number of GI microbial species reaches at least 400, with *Lactobacilli, Streptococci, Clostridia, Bacteroides, Bifidobacteria* and *Escherichia* prevailing (1). Some of these bacteria are also found in cultured milk, yogurt, infant food and pharmaceutical preparations, and are perceived as probiotics because of their presumed beneficial effects on the health of the host. One of the most important features of probiotic microbes is their ability to adhere to the intestinal epithelium which allows sampling and further recognition by the host immune system (2). Adhered probiotic microbes reduce the adherence of pathogenic bacteria strains (3-5). On the other hand, adherence of pathogenic bacteria to the intestinal surface is essential for initiation of infection (6). Thus, adhesion is a key process for survival and colonization of the gut for both probiotic and pathogenic bacteria, involved, respectively, in the health or disease of the host. Bacterial adhesion was intensively investigated with regard to various gastrointestinal physical and chemical factors, *e.g*., pH, oxygenation and digestive secretions (saliva, bile, and gastric, intestinal and pancreatic juices).

Generally, bacterial adhesion has been studied using polymer surfaces (7), intestinal mucus (24, 27, 39), human ileostomy glycoproteins (8), or intestinal cell lines *e.g*. producing mucus HT29-MTX. The Caco-2 cell line, originally isolated from human colon adenocarcinoma (9) in spite of lack of mucus production, is a good *in vitro* model of intestinal epithelium to study bacterial adherence, since the cells can differentiate under standard culture conditions and express characteristics of mature enterocytes, like functional brush border microvilli and apical hydrolases (10-14). Using this model, Darfeuille-Michaud and collaborators (15) have described adhesion of enterotoxigenic *Escherichia coli* (ETEC) strain that produces four different antigen forms of the colonization factor (CFA/I, CFA/II, CFA/III and 2230). Chauviere and collaborators (10) used the Caco-2 cell line as a model for human intestinal epithelium to investigate the effect of *Lactobacillus* spp on pathogen invasion. Other authors have described adhesion to cultured cells of many different *Lactobacillus* (11, 16), *Salmonella* (17) and other bacteria, as well as competition between the microbial species. They also attempted to explain the mechanisms of adhesion and found factors improving the adherence. All these studies substantially have contributed to design probiotics with the best adhesion characteristics.

The mechanism by which GI motility controls bacterial adherence is unclear although the "housekeeping" activity of a migrating motor complex (MMC) is an accepted phenomenon (18). In opposition, pathogenic bacteria

are capable of producing toxins which inhibit gut motility (19, 20). The reduction of gut motility, besides affecting gut metabolism, local blood flow and lymph formation, leads to a marked increase in bacterial adhesion and colonization. However, up to now the two distinct motility phenomena *i.e.*, the electrical activity associated with depolarization of smooth muscle cell membranes (action potentials) and the muscle contractions, were investigated jointly. The action potentials that precede muscle contractions generate the electric fields (EF) (the magnetic field component is negligible) which may be of importance for the microbes living in the intestinal crypts (21). Taking into account the small distance between the colonizing bacteria and intestinal smooth muscle, and the difference in electric charge of bacterial and host cell surfaces (21, 22), we expected that the MMC-related EF may affect bacterial adhesion. Secondly, we thought that this effect might concern mostly the first step of adhesion, *i.e.*, cell-to-cell contact, and much less or not at all the subsequent steps, namely, the affinity and attachment to the receptor. Our previous studies showed that the electric fields produced by the duodenal MMC play a role in bacterial cell division (23) and heat shock proteins (HSP) induction in Caco-2 and bacterial cells (24).

In contrast, the role of pancreatic juice in bacterial attachment remains unknown, although the juice contains a set of enzymatic and non-enzymatic antibacterial proteins (25, 26). Simpson *et al*. (27) in pancreatic insufficiency dogs found that the number of bacteria in duodenal juice increased following pancreatic duct ligation, and then decreased subsequent treatment with exogenous pancreatic enzymes.

The aim of the present study was to estimate the influence of MMC-related EF on adhesion of lactobacilli, lactococci and pathogenic (*Salmonella, Escherichia*) bacterial strains to Caco-2 enterocyte-like cells without and with the presence of pancreatic juice. The source of MMC-related EF was an electromyography signal recorded in the duodenum of a healthy calf, which was reconstructed and transmitted *via* platinum electrodes during *in vitro* experiment involving bacterial and Caco-2 cells (23).

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in *Table 1.* The *Escherichia* coli and *Salmonella enterica* were cultured in Luria-Bertani (LB) broth or on LB plates (LB supplemented with 1.5% agar, Biocorp Ltd, Poland). *Lactobacillus* strains were cultured in MRS broth (de Man, Rogosa, Sharpe) or on MRS plates (MRS broth supplemented with 1.5% agar, Biocorp Ltd, Poland) under anaerobic conditions (in anaerobic jar, OXOID Ltd, UK) at 37°C for 18 - 20 h. A reference strain of *Lactobacillus casei* subsp. *rhamnosus* DSM20021 was cultured as other lactobacilli. *Lactococcus* strains were cultured in M17 medium supplemented with 0.5% glucose or on solid medium additionally supplemented with 1.5% agar under aerobic conditions at 30°C. The number of

bacteria at the beginning of the experiment and those adhering to the Caco-2 cells was expressed as colony forming units (CFU) per ml. The CFUs were determined by plating of 10-fold diluted bacterial suspensions.

Before the experiment, the designated bacterial strain was cultured overnight in an appropriate liquid medium, and subsequently diluted 100-fold in fresh medium (devoid of antibiotics and antimycotics) for culturing Caco-2 cells.

Table 1. List of bacterial strains used in this study

Strain		Relevant characteristic	Reference or source
Gram positive			
DSM20021	*Lactobacillus casei* subsp. *rhamnosus*	Reference strain	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany
IBB2500	*Lactobacillus plantarum*	isolated from cow milk, tet ^{R}	IBB* collection
IBB2588	*Lactobacillus paracasei*	isolated from human stool	IBB* collection
IBB2579	*Lactobacillus casei*	isolated from human stool	IBB* collection
IBB2593	*Lactobacillus casei*	isolated from human stool	IBB* collection
IL594	*Lactococcus lactis* subsp. *lactis*	originally contains plasmids	(11)
IL1403	*Lactococcus lactis* subsp. *lactis*	Plasmid-free derivative of IL594 strain	(11)
Gram negative			
KOS1663	*Salmonella enterica* subsp. *enterica* sv. Enteritidis	isolated from *Salmonella* food-infection area outbreak (1988, Łódź, Poland) from commercial instant soup, bacateriophage type 1 (according to the Lalko phage collection)	National Salmonella Center, Institute of Marine and Tropical Medicine, Gdynia, Poland
269	*Escherichia coli*	O149 : K88; isolated from porcine stool	National Veterinary Research Institute, Puławy, Poland; (37)
259	*Escherichia coli*	O149 : K91; isolated from porcine stool	National Veterinary Research Institute, Puławy, Poland; (36)

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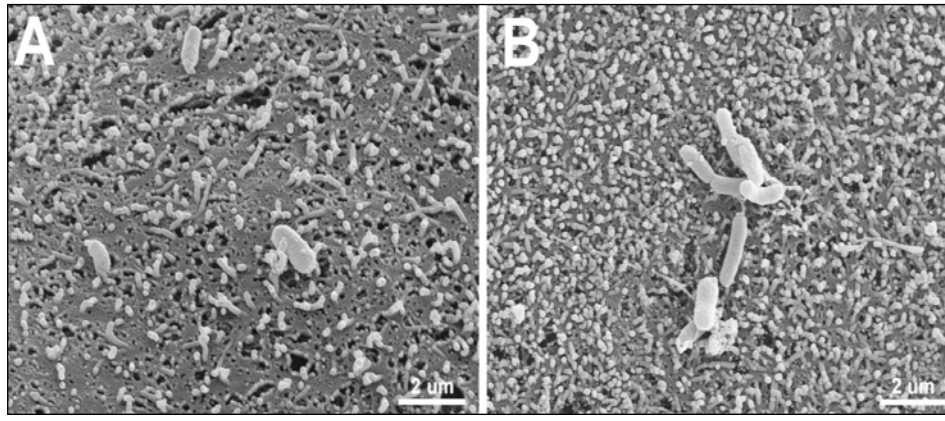

Fig. 1. Scanning electron microscopy photographs of *Salmonella enterica* KOS1663 after 1h incubation with Caco-2 cells: (A) non-differentiated and (B) well-differentiated Caco-2 cells.

Caco-2 cell cultures

Caco-2 cell line (ECACC 86010202) was grown in plastic bottles (25 or 75 cm^2 growth area, Sarstedt, Poland) in standard Dulbecco's modified Eagle's medium (DMEM, IITD PAN, Poland) consisting of 2 mM L-glutamine (Sigma, USA), 1% non-essential amino acids (Gibco, Invitrogen Corporation, USA), 10% heat inactivated foetal bovine serum (Gibco, Invitrogen Corporation, USA), 10 IU/ml penicillin G, 100 µg/ml streptomycin sulphate and 250 ng/ml amphotericin B (all antibiotics and antimycotics were from Sigma, USA). The cells were harvested by trypsinization (0.5% porcine trypsin and 0.2% EDTA in PBS, Sigma, USA) aliquots at 1 x 10^6 cells/ml in the growth medium (described above) supplemented with 10% DMSO (Sigma, USA) and deep frozen at -80°C. For adhesion experiments, fresh DMEM medium was inoculated to the final concentration of 5 x 10^5 cells/ml and added to 2 ml wells with coverslips. To obtain non-differentiated Caco-2 cells (a model of intestinal crypt enterocytes), cultures were grown on coverslips for 6 days. For fully differentiated Caco-2 cells with reconstructed brush border (as a model for villi enterocytes), cultures were grown for 21 days (*Fig. 1*). Cells were cultured in 5% CO_2, 95% relative humidity at 37°C, with medium changed every second day.

Adhesion assay

The coverslips with Caco-2 cells were transferred into Petri dishes, washed twice with PBS, and incubated for 2 and 4 h in fresh DMEM medium (without antibiotics) containing 100-fold diluted bacteria. The sample, but not the control, was exposed to MMC-related EF (see below) *via* two platinum electrodes located in the Petri dish placed in an incubator that provided Faraday shield environment. Experiments were performed at 37°C with gentle rocking (80 rpm/ min). After incubation, cells were washed with PBS and trypsinized as above. Caco-2 cells were counted using a hematocytometer chamber, and the number of adhering bacteria was estimated as described above. Adhesion was expressed as the number of bacterial cells attached to one Caco-2 cell, and confirmed by scanning electron microscopy (*Fig. 1*).

Stimulation with intestinal MMC-related electric fields

A trace of one complete interdigestive duodenal MMC cycle was chosen from a 12 h recording session performed in a healthy 1 month old calf and saved as a data file (time and voltage, MacLab/4e, ADInstruments, Castle Hill, Australia) (28). The average MMC cycle duration was 39±8 min and the contribution of MMC phases was as follows: non-spiking activity (NSA or phase 1) ca. 35%; irregular spiking activity (ISA or phase 2) ca. 55%; regular spiking activity (RSA or phase 3) ca. 10%. This MMC characteristic is similar to that normally observed in the duodenum in suckling human infants (29). The spiking activity (extracted frequency band of 3-50 Hz, amplitude range 500 µV) of one MMC cycle trace was transmitted into the memory of a generating device (SGP-generator, ESCO, Warsaw, Poland) and then retraced *via* platinum electrodes to produce the EF in the *in vitro* experiments (23).

Source of pancreatic juice

Pure inactivated pancreatic juice (PJ) was obtained from conscious weaned pigs (10-25 kg) surgically implanted with a chronic catheter in the accessory pancreatic duct. Animal study protocol was approved by the Local Ethical Committee (26) After a week of recovery the PJ was collected every 2-3 days. Pooled PJ samples collected postprandially were frozen and stored at -20°C until EF-stimulation *in vitro* studies. For adhesion studies, 5 ml of thermo inactivated (15

min at 65°C) PJ was added to 15 ml of fresh DMEM medium. Further procedure was as described in "Adhesion assay" section.

Scanning electron microscopy

Samples of Caco-2 cells with adherent bacteria prepared as described above were fixed in Bouin's solution (Sigma, USA) for 24 h, washed 4 x 10 min in PBS, and dehydrated in a series of ethyl alcohol (10% to 100% in increments of 10% - 10 min each). Alcohol was than replaced with acetone, and samples were at critical point dried (Polarn CPD 7501), sputter coated (Polaron SC 7620) with 30 nm layer of Au-Pd and studied in scanning electron microscope (LEO 1430 VP) at accelerating voltage of 15 kV.

Statistical analysis

The adhesion data were assessed as an average of three independent experiments, each performed in duplicate. Statistical significance was determined by a nonparametric Wilcoxon Rank Sum Test (Mann-Whitney U Test) for unpaired data (KyPlot v.2.0, Koichi Yoshioka) and nonparametric Mann-Whitney test (GraphPad Prism® v. 4.0, GraphPad Software, San Diego, CA, USA). The significance level was set at $P < 0.05$.

RESULTS

Adhesion of various bacterial strains to Caco-2 cells

In our experimental setup the initial number of bacteria, in the range used, had minor, if any, influence on the amount of adhered bacteria. In *Fig. 2*, the adhesion of strains showing more horizontal line between the points denoted less dependency on the initial number of bacteria. The ability of bacteria to adhere to the Caco-2 monolayer varied considerably among the examined strains. In general, all tested lactobacilli showed low adhesion to Caco-2 cells in comparison to other bacterial strains (*Fig. 3*). The lowest adhesion was observed in the case of *L. casei* IBB2579, in which 0.34 bacterium adhered to one Caco-2 cell. The adhesion ratio calculated by dividing the number of adhering bacteria by the original number of bacteria added to the medium, was also the lowest for this strain and equal to 0.21% (*Table 2*). The mean adherence ability of other *Lactobacillus* strains, IBB2588, IBB2593, and IBB2500, ranged from 0.6 to 1.6 bacteria per non-differentiated Caco-2 cell. High attachment ability was observed for *E. coli* 269 isolated from porcine stools (6.3 bacteria per one Caco-2 cell) and pathogenic *S. enterica* KOS1663 (26 bacteria per one Caco-2), although *E. coli* 259 isolated from pigs adhered to the Caco-2 cells at the same level as *e.g.*, *L. casei* IBB2593 (1.73 and 1.62, respectively). Unexpectedly, *Lactococcus lactis* IL594 and *Lactococcus lactis* IL1403 adhered very well to non-differentiated Caco-2 cells (up to 6 bacteria per cell). Interestingly, the time of bacterial contact with Caco-2 influenced the number of adhering lactobacilli and lactococci differently, *e.g.*, for *L. lactis* IL594 a 2-fold better adherence was observed after 2 h of incubation with Caco-2 cells in

comparison to 4 h incubation, whereas for *L. lactis* IL1403 a 4 h incubation resulted in better adherence. In the case of *S. enterica* and *E. coli* 259 the number of adhered bacteria increased almost 5- and 3.5-fold, respectively, after 4 h of incubation in comparison to 2 h incubation.

Bacterial adhesion to differentiated Caco-2 cells

When Caco-2 cells were cultured on coverslips for 6 days, the cells grew as a monolayer and were non-differentiated. In 21-day culture the cells were fully

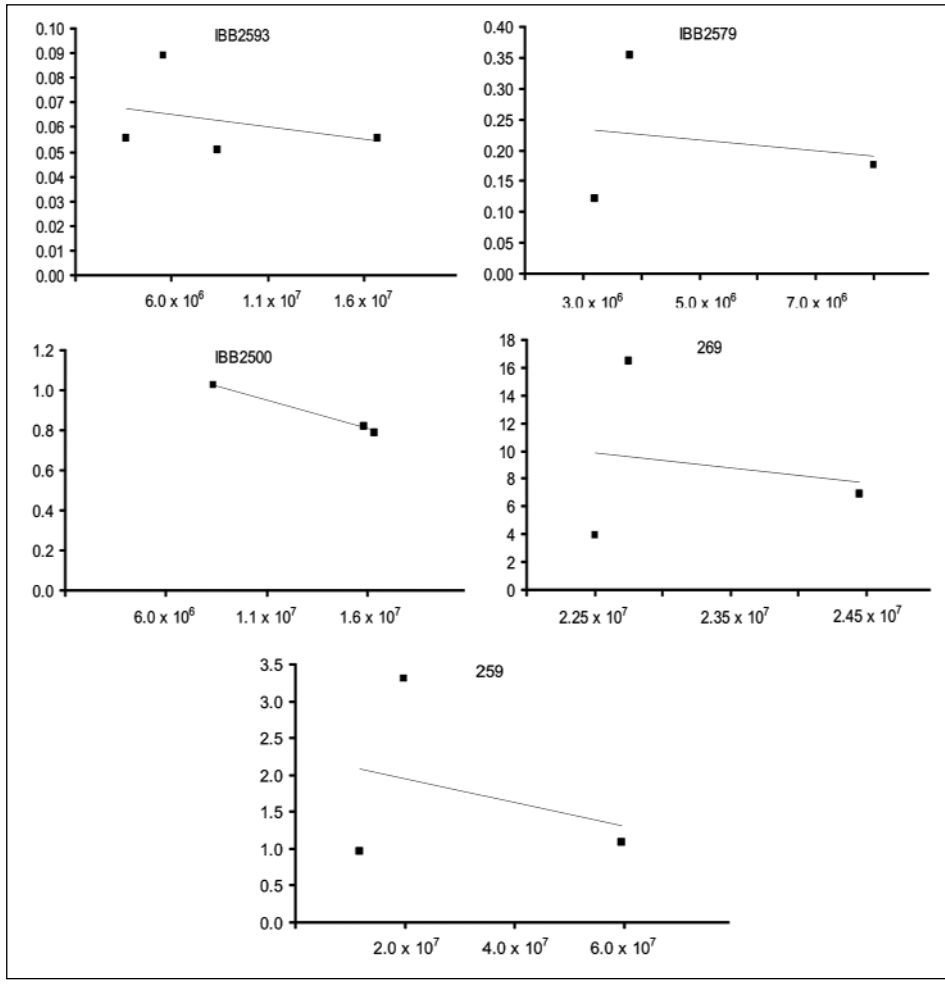

Fig. 2. Dependence of initial number of bacteria on the bacterial adhesion to well-differentiated Caco-2 cells after 4 hours of incubation. Bacterial strains used were as indicated. Y-axis present the number of live, adhering bacteria per one Caco-2 cell, X-axis presents the number of bacteria at the beginning of experiment.

differentiated with reconstructed brush border, better imitating appearance of enterocytes on intestinal villi *in vivo* (*Fig. 1*).

The pathogenic strains used in these experiments (*E. coli* 269 and *S. enterica* KOS1663) demonstrated higher adherence to well-differentiated than to non-differentiated Caco-2 cells (*Fig. 3*). Scanning microscope photographs (*Fig. 1*) showed adhesion of KOS1663 to the brush border created by well-differentiated Caco-2 cells. In the case of this strain, the adhesion ratio during 2 and 4 h experiment was 4-fold higher for well-differentiated (*Table 3*) than for non-differentiated Caco-2 cells (*Table 2*), which corresponds with the adherence results shown in *Fig. 3*. However, for *E. coli* 269 the differences between well- and non-differentiated Caco-2 cells were not statistically significant.

Of the *Lactobacillus* strains tested, IBB2588 and IBB2500 also preferred to attach to well-differentiated cells (*i.e.*, expressing the brush border); however, other strains, like IBB2579 and IBB2593 adhered better to non-differentiated cells. This phenomenon is clearly seen in the case of the latter strain where up to three bacteria were attached to a non-differentiated Caco-2 cell ($P \leq 0.05$, *Fig. 3*),

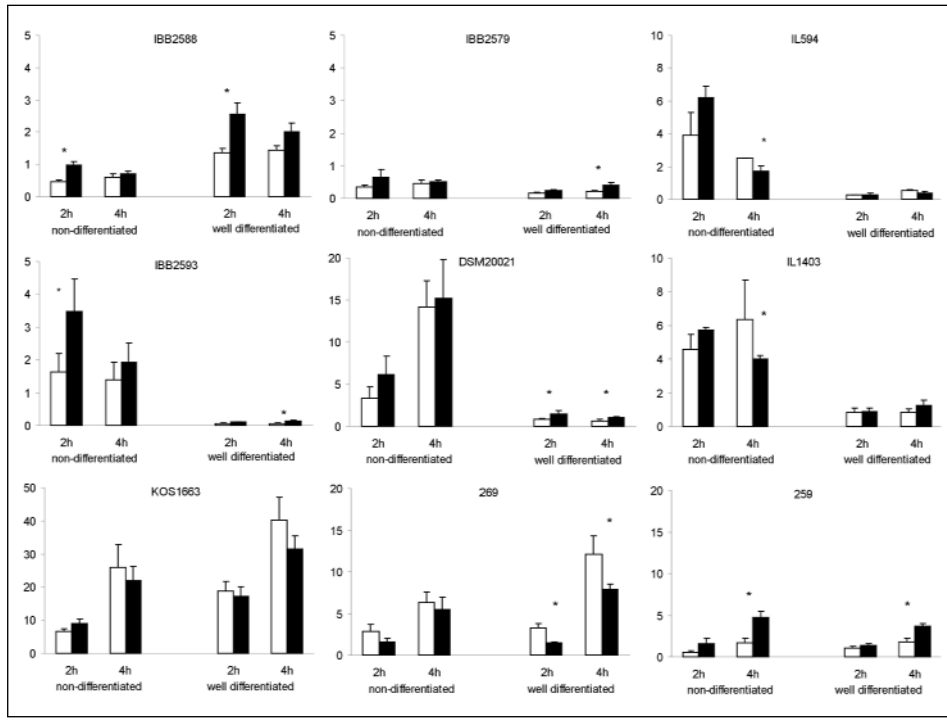

Fig. 3. Bacterial adhesion to Caco-2 cells. White bars - non stimulated cells, black bars - EF stimulated cells. Adhesion assay was done on non-differentiated and well differentiated Caco-2 cells for 2 or 4 h. Bacterial strains used were as indicated. Y-axis present the number of live, adhering bacteria per 1 Caco-2 cell. * indicates the statistical difference, $P<0.05$.

but only about 0.1 bacterium adhered to one well-differentiated cell. The adhesion ratio was not in concert with the above findings since in 2 h experiment with IBB2579 the ratio was 6-fold higher with well-differentiated Caco-2 cells, whereas for IBB2593 it was 4-fold lower. Similar adhesion ratios were observed in the 4 h experiment (*Table 2*).

Two *Lactococcus* strains IL594 and IL1403 adhered much less to well-differentiated Caco-2 cells than to non-differentiated ones (20- and 5-fold, respectively, $P \leq 0.01$).

The effect of MMC-related EF on bacterial adhesion to Caco-2 cells

Exposure of bacteria and Caco-2 cells to the electric fields associated with duodenal MMC resulted in different efficiency of bacterial adhesion to Caco-2

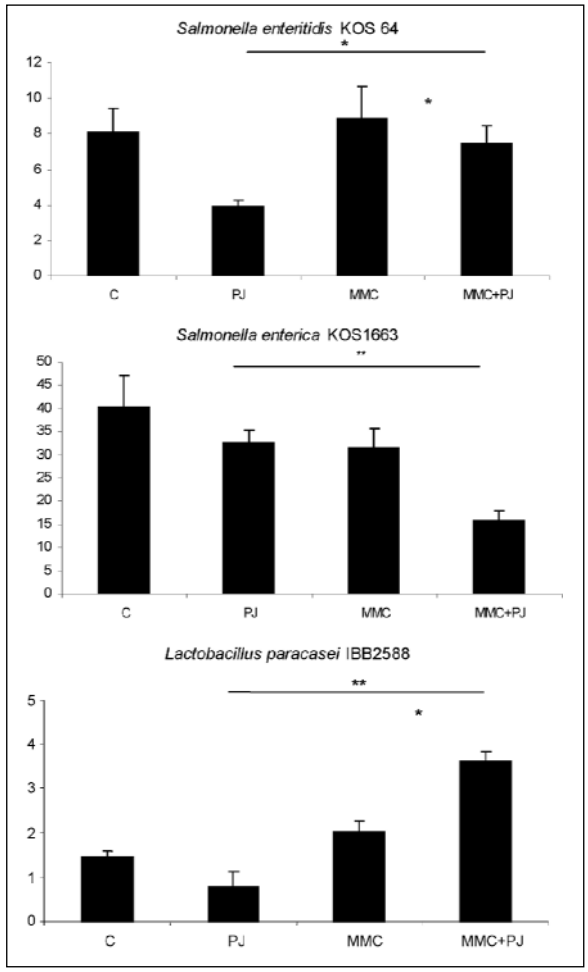

Fig. 4. Bacterial adhesion to Caco-2 cells stimulated or not (C) with MMC-related EF (MMC), in the presence of porcine pancreatic juice (PJ), or both factors (MMC+PJ). Adhesion assay was done on well differentiated Caco-2 cells for 4 h. Y-axis - number of live, adhering bacteria per one Caco-2 cell. * $P<0.05$; ** $P<0.01$

cells. In general, lactobacilli adhered better to EF-stimulated Caco-2 although efficiency of this stimulation was depended on the degree of Caco-2 cells differentiation. Following 2 and 4 hour incubation of the reference strain *L. rhamnosus* DSM20021 with well differentiated Caco-2 cells, 0.85±0.14 and 0.66±0.15 bacterial cells, respectively, adhered to one Caco-2 cell. The EF stimulation significantly enhanced ($P<0.05$) the number of adhering bacterial cells

Table 2. Adherence of bacteria to non-differentiated Caco-2 cells stimulated or non-stimulated with the Mioelectrical Migrating Complex - related electric field.

Strain		Bacterial adhesion ratio (%)			
		Adhesion performed for 2 h		Adhesion performed for 4 h	
		non-stimulated	EF-stimulated	non-stimulated	EF-stimulated
IBB2588	*L. paracasei*	1.50±0.92	2.69±1.20	1.24±0.25	1.85±0.87
IBB2500	*L. plantarum*	1.31±0.34	2.30±1.01	0.72±0.07	1.15±0.33*
IBB2593	*L. casei*	0.86±0.71	1.39±1.06	0.62±0.64	0.67±0.44
IBB2579	*L. casei*	0.21±0.14	0.37±0.23	0.26±0.20	0.27±0.15
KOS1663	*S. enterica*	2.02±0.37	2.23±0.79	5.54±2.15$^{\#\#\#}$	4.90±0.67$^{\#\#\#}$
259	*E. coli*	0.75±0.45	1.80±0.92	2.05±0.51$^{\#\#\#}$	5.12±1.05$^{**\,\#\#}$
269	*E. coli*	2.35±0.54	1.97±0.87	6.07±2.79$^{\#\#}$	5.75±1.92$^{\#}$
IL594	*L. lactis*	0.73±0.56	1.39±0.79	1.04±0.44	0.51±0.31
IL1403	*L. lactis*	0.73±0.08	1.04±0.15*	1.46±0.75	0.83±0.31

The adhesion ratio was calculated by dividing the number of live, adhering bacteria by the number of bacteria added to the medium at the beginning of the experiment. Asterisks indicate statistical difference between the non- and EF-stimulated bacterial cultures (* $P\leq0.05$; ** $P\leq0.01$); # indicates statistical difference between bacteria cultured for 2 and 4h, respectively (# $P\leq0.05$; ## $P\leq0.01$; ### $P\leq0.001$).

Table 3. Adherence of bacteria to well-differentiated Caco-2 cells stimulated or non-stimulated with Mioelectrical Migrating Complex - related electric field.

Strain		Bacterial adhesion ratio (%)			
		Adhesion performed for 2 h		Adhesion performed for 4 h	
		non-stimulated	EF-stimulated	non-stimulated	EF-stimulated
IBB2588	*L. paracasei*	1.29±0.35	2.31±0.79*	1.67±0.42	1.82±0.57
IBB2500	*L. plantarum*	2.14±0.86	1.78±0.94	1.80±0.48	2.08±0.57
IBB2593	*L. casei*	0.20±0.11	0.32±0.12	0.20±0.09	0.40±0.21
IBB2579	*L. casei*	1.36±0.84	2.18±1.16	1.79±1.25	2.51±0.74
KOS1663	*S. enterica*	8.88±3.19	8.74±2.13	19.25±6.81	16.14±6.66$^{\#}$
259	*E. coli*	0.61±0.35	1.10±0.73	0.88±0.42	2.48±1.29$^{*\,\#}$
269	*E. coli*	1.67±0.41	2.25±1.25	5.27±3.90	4.65±1.47
IL594	*L. lactis*	0.22±0.07	0.27±0.13	0.41±0.24	0.34±0.18
IL1403	*L. lactis*	1.94±2.24	1.58±1.18	1.00±0.41	1.59±0.60

The adhesion ratio was calculated by dividing the number of live, adhering bacteria by the number of bacteria added to the medium at the beginning of the experiment. Asterisks indicate statistical difference between the non- and EF-stimulated bacteria cultures (*$P\leq0.05$); # indicates statistical difference between bacteria cultured for 2 and 4h, respectively (# $P\leq0.05$).

(1.49±0.42 and 1.06±0.11, respectively). *L. paracasei* IBB2588 adhered 2-fold better to EF-stimulated Caco-2 cells, non-differentiated and well-differentiated, but only in the 2 h experiment (*Tables 2* and *3*). In 4 h experiment the differences between EF-stimulated and non-stimulated control were not statistically significant. *L. casei* IBB2593 adhered 2-fold better to 2 h stimulated non-differentiated cells and also to 4 h stimulated well-differentiated cells. *L. casei* IBB2579 reacted in the same way as *L. casei* IBB2593. On the contrary, adhesion of *L. plantarum* to non-differentiated cells was weakly stimulated by EF in 2 h experiment (1.5-fold), but not stimulated at all in 4 h experiment; with well-differentiated Caco-2 there was no stimulation in either 2 or 4 h experiments (*Fig. 3*). There was no influence of EF stimulation on the adhesion of two *L. lactis* strains, IL1403 and IL594.

Interestingly, pathogenic *S. enterica* KOS1663 adhering very strongly to Caco-2 cells showed reduced adhesion during EF stimulation (respectively, 40 vs. 31 bacteria/Caco-2 cell, $P<0.01$, *Fig. 3*). Similarly, in experiments with *E. coli* 269 there was an almost 2-fold decrease in adhesion to EF-stimulated Caco-2 cells ($P\leq0.05$, *Fig. 3*) despite no significant changes in the adhesion ratio in non-differentiated *vs*. well-differentiated cells, and 2 *vs*. 4 h incubation (*Tables 2* and *3*). In the case of the *E. coli* 259 strain, a statistically significant increase (about 3-fold) of adhesion to EF-stimulated, non-differentiated Caco-2 cells was observed after 4 h of incubation ($P\leq0.05$, *Fig. 3*). Accordingly, a 3-fold increase was also observed in the adhesion ratio for this strain (*Table 2*).

The effect of pancreatic juice on bacterial adhesion to Caco-2 cells

Three bacterial strains were used for adherence studies employing pancreatic juice, namely, *L. paracasei* IBB2588, *S. enterica* KOS 1663, and *E. coli* 269. The potency of adhesion of these strains to Caco-2 cells was measured in the presence of PJ and also when both factors, PJ and MMC-related EF were acting on bacteria and Caco-2 cells. The results presented in *Fig. 4* show that PJ (without EF presence) decreased adhesion of all three strains tested. However, simultaneous action of PJ and EF increased over 2-fold the adherence of *Lactobacillus* in comparison to untreated control (*i.e.*, in the absence of PJ and EF), whereas pathogenic *Salmonella* adhered over 2-fold less efficiently under the same conditions as compared to untreated control. Interestingly, PJ alone stronger inhibited the adhesion of *E. coli* 269 to Caco-2 cells than the exposure to EF alone or a combination of the two (PJ and EF).

DISCUSSION

It is well known that the reduction of intestinal motility and secretion of pancreatic juice are immediately followed by intestinal bacteria overgrowth but the mechanisms involved in their attachment and colonization are not perfectly clear. Our *in vitro* study demonstrates for the first time that the electric field (EF), a

myoelectric component of the intestinal MMC as well as the pancreatic juice are able to change bacterial adhesion to Caco-2 cells fixed on a glass surface. Moreover, the adhesion to Caco-2 cells in most instances differs depending on the degree of cell differentiation. Under normal grow conditions, Caco-2 cells attach to a glass surface or mesh gradually and form a confluent monolayer of polarized cells, displaying an intestinal enterocyte-like structure, *i.e.*, tight junctions, an apical membrane with microvilli (*Fig. 1B*) and a baso-lateral membrane. Simultaneously, the cells express apical hydrolases and a polarized transport system, *e.g.*, Na^+-dependent co-transporters (30) and Cl^- secretion (13, 14, 31). All these features make Caco-2 cells a good, however not perfect (lack of mucus production) *in vitro* model to study bacterial adhesion in the gut.

Without any electrical stimulation the adhesion capacity of *Lactobacillus* strains growing in Faraday cage is weak (less than one bacterium per Caco-2 cell) in comparison to the remaining strains tested. Though lactococci adhere to Caco-2 cells much better than lactobacilli, they are still considered to be non-colonizing bacteria that remain in the GI tract lumen only transiently (32). Both *Lactococcus* and *L. casei* strains adhere better to non-differentiated Caco-2 cells than to well-differentiated ones (*Fig. 3*, *Tables 2* and *3*). This is in agreement with the previous *in vivo* studies showing the colonization of lactobacilli mainly in the intestinal crypts, *i.e.*, on the epithelial cells that undergo differentiation, whereas the pathogenic bacteria encounter the upper part of intestinal villi and thus adhere to well-differentiated enterocytes (fimbrial or membrane type of adhesion) (33).

For many bacterial pathogens adherence to the GI epithelial cells is a critical step, since the adhering bacteria can release enzymes and toxins, and trigger changes in the target cell facilitating the invasion (34, 35). Here, we present the adhesion capacity of known pathogen *S. enterica* and two *E. coli* strains, 259 and 269, isolated from diarrhea. After 4 h of contact more than 25 *S. enterica* cells attached to a non-differentiated Caco-2 cell and over 40 to a well-differentiated ones. Among the two *E. coli* strains, the 269 strain behaved similarly to *S. enterica* (over 6 and 12 bacteria adhering to non-differentiated and well-differentiated Caco-2 cells, respectively) whereas the 259 strain, with its low adhesion capacity (less than 2 bacteria per Caco-2 cell) and no differences between adhesion to non-differentiated and well-differentiated Caco-2 cells, behaved rather like *L. plantarum* IBB2500 (*Fig. 3*). Both *E. coli* strains contain the same O antigen (O149) although the K antigen is different. The *E. coli* 259 contains a non-adhesive antigen K91 (36), whereas *E. coli* 269 possesses a colonization factor K88, which is a fimbrial antigen of critical importance for bacterial adherence (37).

The influence of electric fields generated by the gut on bacterial cells has been described earlier (38, 39). Previously, we have proved that MMC-related EF enhance *E. coli* growth in bacterial culture (31) and induce heat shock response (24). In the present study, we showed not only the differences in the adhesion

capacity between lactobacilli and pathogenic, Gram-negative bacteria, but also different reactions of these strains to EF stimulation. Pathogenic *S. enterica*, adhering to Caco-2 extremely well, reduced its adherence in the presence of intestinal MMC-related EF. On the contrary, lactobacilli, which adhered with about 20-fold lower efficiency than *S. enterica*, showed at least 2-fold better adhesion in the presence of the EF.

The ability of bacteria to adhere to intestinal cells has been one of the criteria for selection of probiotic strains (3, 40). Considering strong adhesion of bacterial pathogens (regarded apparently unbeneficial for the host), the improvement in adhesion capacity of lactobacilli by intestinal EF stimulation is regarded beneficial and of considerable importance. The enhanced adhesion of probiotic bacteria gives them a better chance to force out bacterial pathogens and colonize, even transiently, the GI tract epithelium. Besides pathogen elimination, the colonization increases the survival of probiotics, which is another beneficial *in vivo* effect. Accordingly, our recent *in vitro* study with *S. enterica* and *L. casei* submitted together to Caco-2 cell culture showed a dramatic reduction in *S. enterica* adhesion in contrast to only a 2-fold decrease in *L. casei* adhesion (41). Therefore, the EF associated with the physiological motility of the gut seems to play an important role in establishing microbial homeostasis of the gut.

We have also shown that porcine pancreatic juice can modulate the adherence of both probiotic, conditionally pathogenic and pathogenic bacteria to the gut epithelium. In general the pattern was similar to that observed following exposure to the intestinal MMC-related EF. The factor, or factors in pancreatic juice, however, were not isolated since it was beyond the scope of the present study. Nevertheless, it seems that it is not any of pancreatic digestive enzymes since we inactivated them by heating the juice before adding it to the cell medium. Previously, we have found and partially isolated another thermoresistant protein of antibacterial activity (25, 26), but the protein was active in pH 8 or higher. No specific studies on the role of pancreatic juice on bacteria adherence are available, though Simpson *et al* (27) found in pancreas insufficiency dogs an increased gastrointestinal bacteria number in duodenal fluid.

To conclude, the intestinal MMC-related EF as well as the pancreatic juice can modulate the adhesion of bacteria existing in the gut or temporarily passing through the intestine. However, as we have shown in our *in vitro* experiments, their influence on the adhesion is different depending on bacterial species. MMC-related EF and pancreatic juice act against adhesion of Gram-negative bacterial pathogens and positively modulate the adhesion of Gram-positive lactic acid bacteria. The *in vitro* approach proposed in our study may serve as a simple tool for selecting probiotics among lactic acid bacteria characterized with the best adhesion properties. If the described phenomenon exists *in vivo*, the modulation of adhesion capacity of gastrointestinal and pathogenic bacteria by the myoelectrical activity of small intestine and pancreatic juice may be, on

the one hand, a defensive mechanism against infection, and on the other, a mechanism counteracting probiotic elimination by increasing their adhesion to epithelial cells of GI tract.

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