

Cellular differentiation of non-transformed intestinal epithelial cells is regulated by *Lactobacillus rhamnosus* and *L. casei* strains

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Summary

The aim of this study was to characterize an *in vitro* modulating effect of three commensal *Lactobacillus* strains on cellular differentiation of non-transformed crypt-like rat small intestinal cell line IEC-18. IEC-18 was grown on extracellular matrix (ECM), with or without presence of *Lactobacillus* strains. Gene expression of IEC-18 bacterial detection system - such as Toll-like receptors TLR-2, TLR-4, signal adapter MyD88, cytoplasmic NOD2 receptor, inflammatory cytokines IL-18, IL-1 β , chemokine IL-8 and enzyme caspase-1 - was evaluated using real-time PCR. Expression and localization of TLR-2, TLR-4, IL-18 and caspase-1 proteins was demonstrated by Western blotting and immunofluorescent staining. Secretion of IL-18 to apical and basolateral surfaces was assayed by ELISA. Our results suggested that *L. casei* LOCK0919 accelerated differentiation of IEC-18 by stimulating TLR-2, TLR-4, MyD88, IL-18, caspase-1 mRNAs and proteins. *L. casei* LOCK0919 increased expression and transfer of villin and β -catenin from cytoplasm to cell membrane. Presence of *L. rhamnosus* LOCK0900 resulted in detachment of IEC-18 layer from extracellular matrix leading to induction of IL-1 β , of TLR-2 and IL-8 mRNAs and stimulation of MyD88, caspase-1 and cytosolic receptor NOD2 mRNAs. *L. rhamnosus* LOCK0908 was not recognized by TLR-2 or TLR-4 receptors. Lactobacilli-IEC-18 crosstalk enhanced immune and barrier mucosal functions.

Key words

IEC-18 · *Lactobacillus* · IL-18 · TLRs · caspase-1 · villin · β -catenin

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Introduction

The epithelial surface of the small intestine is separated from the intestinal lumen by a single epithelial cell layer constantly migrating along the crypt/villus axis within 3-5 day cycle from the proliferative undifferentiated crypt cells to the fully differentiated polarized columnar enterocytes with apical and basolateral membrane domains. The enterocytes are the most abundant cell type which together with Paneth and goblet cells contribute to mucosal defense and maintenance of barrier integrity (Ismail and Hooper 2005). Intestinal epithelial cells (IEC) provide their own control of proliferation and differentiation of cell functions. Presence of non-differentiated stem cell precursors permits epithelial self-renewal. The actin cytoskeleton plays the major role in cell migration, cell-to-cell adhesion and also in cell proliferation and differentiation (Heath 1996). IEC exist as adherent cells attached to a basement membrane consisting of various extracellular matrix proteins (ECM). The molecular conformation of ECM, such as laminin and collagen, modulate the epithelial cell phenotype (Kedinger 1994). Incubation *in vitro* of nonmalignant IEC on ECM - Matrigel induces changes in IEC phenotype toward full differentiation, such as expression of digestive brush-border enzymes (Sanderson *et al.* 1996, Kolinska *et al.* 2008) and regulation of IEC polarity (Jalal *et al.* 1992). The short life cycle of enterocytes is terminated by physiological apoptosis/shedding after their gradual detachment from ECM proteins.

The IEC monolayer serving as the interface between the host and environment is constantly exposed to bacterial content in the gut lumen. IEC possess receptors for molecules derived from bacterial cell wall. Toll-like transmembrane receptors (TLR) are a key group of pattern recognition receptors; DNA transcription generates their mRNAs that carry the information for protein synthesis. TLR-2 and TLR-4 mRNAs had been detected in human colon cancer cell lines T84 and HT-29 but proteins were weakly detectable (Stadnyk *et al.* 2001). However, surface protein expression of TLR-2 of 85 kDa and TLR-4 of 92 kDa was

visualized on T84 cells (Cario *et al.* 2000). Synthesis and insertion of transmembrane receptors to plasma membrane operate according to hypothesis of Blobel (Blobel *et al.* 1979); the integral protein destined for the plasma membrane is synthesized on polyribosomes and its signal peptide enables attachment to rough endoplasmic reticulum (ER) and travel from ER to the site of complex glycosylation through the Golgi and trans-Golgi network, the protein then fuses with plasma membrane. Demonstration that TLR-2 and TLR-4 exist on normal human IEC helped explain the capacity of human enterocytes as non-classical immune cells to function as active participants in immune response and physical barrier to bacterial substances (Fusunyan *et al.* 2001). The second major group of pattern recognition receptors is a nucleotide-binding domain (NOD) family, a group of cytoplasmic proteins that does not contain a transmembrane domain, does not act as membrane receptor (Barnich *et al.* 2005) and does not contain a signal peptide. The authors assumed that NOD2 interacts with the inner side of the plasma membrane necessary for NOD2 to recognize its bacterial ligand muramyl dipeptide and to activate nuclear factor kappa B (NF- κ B).

Probiotics are living, non-pathogenic microorganisms that colonize the intestine and provide benefit to the host. In this study, we have tested the effect of three probiotic *Lactobacillus* strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 isolated from human fecal samples and known for their anti-allergic ability (Cukrowska *et al.* 2010, Kozakova *et al.* 2016). We have used a model of non-transformed undifferentiated small intestinal cell line IEC-18 derived from the rat ileum that undergoes differentiation during cultivation on ECM similarly as IEC-6 line (Kolinska *et al.* 2008). We intended to characterize the modulating effect of probiotic bacteria on immunological and morphological functions during differentiation. Activation of TLRs by commensal bacteria is essential in maintaining intestinal epithelial homeostasis (Michelsen and Arditi 2007). Thus, gene expression of TLR-2, TLR-4 and internal NOD2 receptor activation at mRNA level have

been evaluated. Next we followed MyD88, an adaptor functional molecule critical for TLRs-mediated signaling and transcription of chemokine IL-8. Expected modulation of TLR protein glycosylation by commensal bacteria have been correlated with the degree of cell differentiation. Furthermore, we focused on the effect of *Lactobacillus* strains on gene expression of inflammatory cytokines, interleukin IL-18 and IL-1 β , sharing a common maturation mechanism that requires intracellular enzyme caspase-1. Previously, we have shown a correlation between constitutive expression of IL-18 and caspase-1 proteins (Kolinska *et al.* 2008). Leaderless proteins IL1- β and IL-18 are actively secreted by human monocytes and dendritic cells, respectively, via a pathway of secretion different from the classical ER-Golgi pathway. Instead, secretion operates at the level of both proteolytic processing and extracellular export and may be increased by stress conditions (Rubartelli *et al.* 1990, Gardella *et al.* 1999). In this study, we are also characterizing subcellular localization of cytoskeleton binding proteins villin and β -catenin in course of IEC differentiation and effect of lactobacilli.

Materials and Methods

Bacterial strains

L. rhamnosus LOCK0900 (L900), (Aleksandrak-Piekarczyk *et al.* 2013) *L. rhamnosus* LOCK0908 (L908) (Koryszewska-Baginska *et al.* 2014) and *L. casei* LOCK0919 (L919) (Koryszewska-Baginska *et al.* 2013) were obtained from the Pure Culture Collection of the Technical University of Lodz, Poland (LOCK). Overnight cultures in MRS broth (Oxoid, Basingstoke, UK) were centrifuged, washed in sterile phosphate-buffered saline (PBS) and

concentration was adjusted to 10^9 CFU/ml. Single bacterial strains were inactivated with 1 % formaldehyde-PBS for 3 h at room temperature, washed twice with sterile PBS and stored at – 20 °C. Each bacterial strain was applied to IEC-18 cells at 10^7 CFU/ml for 2 h either separately or in an equal-part mixture (Lac).

IEC-18 cultures

The non-transformed rat intestinal epithelial cell line IEC-18 derived from ileum was obtained from American Type Culture Collection (ATCC, CRL-1592, Manassas, VA). The stock culture was grown in Dulbecco modified Eagle medium (DMEM) in 95 % air, 5 % CO₂ at 37°C. The medium contained 40 µg gentamicin/ml, 2 mM glutamine, 50 ml fetal bovine serum (FBS)/l and 10 mg insulin/l (Kolinska *et al.* 2008). Passages 15-27 were used in this study. For real-time PCR and Western blot the cells were plated at a density of 1.6×10^6 /cm² onto plastic plates (60 mm in diameter) coated with Matrigel (Engelbreth-Holm-Swarm sarcoma basement membrane, Sigma, St. Louis, MO). For IL-18 bipolar secretion and cell accumulation assay (ELISA, Biosource Internat., Nivelles, Belgium) the cells were seeded on either non-coated or collagen coated 6.5 mm, 5 µm pore-size Costar Transwell filter inserts (Corning Corporation, Corning, NY) in a two-compartment system. The cultivation medium contained a mixture of five nucleotide monophosphates (10 mg/l each) added on the second day after seeding. On day six the medium was changed to a medium with a lower amount of FBS (5 ml/l) for 24 hours. On day seven formalized bacteria (10^7 CFU/ml) were added for two hours before the harvest. Culture supernatants were collected and stored at -75 °C. Cells were harvested by gentle trypsinization with 0.25 % trypsin - 0.3 % EDTA, washed with PBS and, after estimating the cell counts, pelleted at 10 000g. The pellets were homogenized 1) in lysis buffer RLT for RNA isolation or 2) in lysis buffer for Western blot and ELISA (Vallee *et al.* 2004) containing protease inhibitor cocktail (Sigma) and 1µM each of aprotinin,

leupeptin and pepstatin. Cell lysates were centrifuged at 15 000 x g for 10 min at 4 °C and the supernatants stored at – 75 °C after assaying their protein content.

Western blotting

Supernatants obtained after homogenization of cell pellets in lysis buffer of protein concentration 1.5 mg/ml were separated on the 5-20 % SDS-PAGE under reducing conditions. Separated proteins were transferred onto nitrocellulose membranes blocked with Tris-buffered saline with 0.1% Tween 20 (TBS) (Golias *et al.* 2013). Blocked membranes were incubated with a primary antibody (Ab) diluted in TBS containing 0.1 % defatted milk (Promil, Nový Bydžov, CR) for 1 h at room temperature. Primary Abs: goat anti-IL-18 diluted 1:5000, goat anti-TLR-2 and goat anti-TLR-4, both diluted 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); Control recombinant rat IL-18 protein (R&D Systems, Minneapolis, MN); rabbit anti-caspase-1 (Cell Signaling Technology, Danvers, MA) diluted 1:1000. The membranes were incubated with peroxidase-conjugated secondary Ab: donkey anti-goat IgG, goat anti-rabbit IgG (Cell Signaling Technology) diluted 1:5000 with TBS containing 1 % milk. Signal was detected using the West Femto Maximum Sensitivity Substrate kit (Pierce, Rockford, IL) and exposed onto films (Santa Cruz Biotechnology).

RNA preparation and Real-time PCR

Total RNA was isolated from IEC-18 cells using the RNeasy Plus Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer's protocol. RNA concentration was quantified by ultraviolet spectrophotometry at 260 nm, and the purity and integrity were determined using a NanoDrop (Thermo Fisher Scientific, Wilmington, DE). Real-time (RT)-PCR assays were performed to quantify steady-state mRNA levels of IL-8 (CXCL1), caspase-1 (Cas-1), IL-1 β , IL-18, myeloid differentiation primary response gene 88 (MyD88), nucleotide oligomerization domain (NOD) 2, TLR-2, and TLR-4. cDNA was synthesized from 0.2 μ g of

total RNA. RT-PCR amplification was performed using Primer Express Software™ (Applied Biosystems, Foster City, CA). Target probe was labeled with fluorescent reporter dye. PreDeveloped TaqMan primers and probes were used for the detection of IL-8, Cas-1, IL-1 β , IL-18, MyD88, NOD2, TLR-2, and TLR-4. Reporter dye emission was detected by an automated sequence detector combined with ABI Prism 7700 Sequence Detection System® software (Applied Biosystems). RT-PCR quantification was then performed using TaqMan® 18S controls.

Immunofluorescence

The rat IEC-18 cells were plated on non-coated round coverslips (30 mm in diameter) in plastic plates with DMEM-based medium as reported previously. IEC-18 cells were either untreated or treated with *L. casei* LOCK0919 (10^7 CFU/ml) for 2 h before the end of cultivation. After cultivation for 1, 3, 5 or 7 days, the cells were fixed with cold methanol (-25 °C) for a minimum of 1 h and washed three times with PBS. Non-specific binding of IgG was suppressed with 3 % BSA for 45 min. To increase the permeability of cell membranes, 0.1 % Triton X-100 was added before the overnight incubation with primary antibody (Ab) at 4 °C. Primary Abs: goat anti-villin, goat anti-TLR-2, goat anti-TLR-4 (Santa Cruz Biotechnology, Santa Cruz, CA) all diluted 1:50. Rabbit anti- β -catenin (Sigma, St. Louis, MO) diluted 1:300. Rabbit anti-caspase-1 (Gen Way Biotech, San Diego, CA) ready-to-use. Rabbit anti-IL-18 (Ab Bio Technology, Miami, FL). Secondary antibodies used: donkey anti-goat IgG-FITC (Santa Cruz Biotechnology), and donkey anti-rabbit IgG-TRITC (Jackson Immuno Labs, West Grove, PA), both diluted 1:100 and incubated for 45 min at room temperature. Mounting medium was Pro Long Anti-fade Gold with 4, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) as nuclear marker. The samples were viewed under an Olympus Provis AX 70 microscope equipped with Olympus DP 70.

Statistics

Statistical analysis of differences between groups of ELISA values was evaluated by one-way analysis of variance (ANOVA). The unpaired Student's t-test was used for relative mRNA levels. Statistical analysis was performed using Graph Pad Prism 5.0 Software (GraphPad Software, Inc., San Diego, CA). All numerical data expressed as mean \pm SEM. $P < 0.05$ was considered to be significant.

Results

Bipolar IL-18 secretion by IEC-18 cells in response to apical lactobacilli mixture (*L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, *L. casei* LOCK0919)

For IL-18 measurements (ELISA), the IEC-18 cells were seeded either on non-coated or collagen-coated microporous transwell filter inserts in DMEM media in both upper (apical) and lower (basolateral) chambers. The cells were grown for 7 days to achieve their polarity. In collagen-free system (-collagen), the cells grew as less differentiated crypt-like small intestinal epithelial cells. More differentiated villus-like IEC-18 cells were observed on microporous insert coated with collagen (+collagen). The equal-part mixture (10^7 CFU/ml each) of three *Lactobacillus* strains (Lac) was added to the upper chamber of the transwell plates for the last 2 h of cultivation. Control crypt-like IEC-18 secreted constitutive amounts of IL-18 into both apical and basolateral chambers. Control villus-like IEC-18 secreted higher amounts of IL-18 compared to crypt-like IEC-18 cells. Cultivation of IEC-18 cells with Lac mixture significantly reduced the IL-18 secretion in villus-like epithelial cells (Fig. 1A). Intracellular content of IL-18 in IEC-18 cells layer increased after bacterial challenge, both without/with collagen presence (Fig. 1B).

***L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 differentially influence mRNA production of TLR-2, TLR-4, NOD2 receptor and adaptor molecule MyD88 important for TLR signal pathways to activate NF- κ B**

Our results in Fig. 2 showed strong induction of TLR-2 ($P < 0.001$) and NOD2 ($P < 0.05$) relative mRNA levels after cultivation of IEC-18 cells with *L. rhamnosus* LOCK0900. In contrast to *L. rhamnosus* LOCK0900 strain, gene expression of NOD2 was not affected by *L. casei* LOCK0919 treatment. However, significant up-regulation of TLR-2 ($P < 0.05$) and a mild insignificant stimulation of TLR-4 were observed after cultivation with *L. casei* LOCK0919 strain. Both probiotic *L. rhamnosus* LOCK0900 and *L. casei* LOCK0919 have in common an insignificantly increased expression of mRNA MyD88 functional molecule. No effect of *L. rhamnosus* LOCK0908 strain either on TLR-2, TLR-4 or NOD2, MyD88 relative mRNA levels was observed. Western blotting using relevant antibodies showed constitutively expressed TLR-2 and TLR-4 proteins in control group of 100-105 kDa, which is higher than those shown on human fetal primary small intestinal cell line 85 kDa for TLR-2 and of 92 kDa for TLR-4 (Fusunyan *et al.* 2001).

***Effect of L. casei* LOCK0919, *L. rhamnosus* LOCK0900 and *L. rhamnosus* LOCK0908 on the expression of mRNA of the enzyme caspase-1, cytokines IL-1 β , IL-18 and IL-8 in IEC-18 cells**

Caspase-1 is a cysteine protease which cleaves inflammatory cytokines pro-IL-18 and pro-IL-1 β into their active mature forms. Our results showed that caspase-1 and IL-18 mRNAs were significantly increased after cultivation of IEC-18 cells with *L. casei* LOCK0919 strain (Fig. 3A). In contrast to *L. casei* LOCK0919, gene expression of caspase-1 was not significantly stimulated and gene expression of IL-18 was not affected after cultivation with *L. rhamnosus* LOCK0900 strain. However, significant up-regulation of IL-1 β mRNA and IL-8 mRNA

appeared after cultivation with *L. rhamnosus* LOCK0900 strain. IL-8 mRNA is a transcription product of TLR-2 activation by bacteria. No effect of *L. rhamnosus* LOCK0908 strain on studied cytokines was observed. Western blotting (Fig. 3B) using rabbit anti-caspase-1 antibody showed six mature active caspase-1 protein forms between 37-53 kDa in control group and in the *L. casei* LOCK0919 treated IEC-18 cells. The three bands in the range of 6-18 kDa of IL-18 obtained after reaction with rabbit antibody to IL-18 may represent pro-IL-18 and also mature IL-18 protein forms correlated with the location of recombinant rat IL-18.

Immunofluorescent localization of proteins TLR-2, TLR-4, caspase-1 and IL-18 on IEC-18 cells: exposure to L. casei LOCK0919

Immunofluorescent staining was performed on IEC-18 cells cultivated for 7 d on glass in the absence of ECM to get a better view of independent effect of bacteria. After IEC-18 cells were exposed to *L. casei* LOCK0919, an increase of immunofluorescence of TLR-2, TLR-4, caspase-1 and IL-18 proteins was more pronounced in the cytoplasm of IEC-18 cells in comparison with control cytoplasm without bacteria (Fig. 4).

L. casei LOCK0919 modulated villin and β -catenin expression in IEC-18 cells: immunofluorescence study

Villin is a tissue specific actin-binding protein expressed in microvilli in the brush borders of enterocytes. It participates in the assembly of the intestinal brush-border cytoskeleton and in the maintenance of brush-border architecture (Athman *et al.* 2002) . Our results presented in Fig. 5 clearly showed the stimulating effect of Matrigel on the expression of villin in the IEC-18 cells cultivated for 5D (5 days). (B). Villin is synthesized in small amount without Matrigel support (A). To eliminate the effect of ECM in studies with probiotic bacteria, the IEC-18 cells were cultivated on plates without Matrigel. On cultivation for 3D (C) and 7D (E) the

expression of villin in control culture did not substantially differ from that at 5D in even distribution in the cytoplasm. Cultivation of IEC-18 cells with *L. casei* LOCK0919 strain stimulated villin expression on 3D to form spherical structures thicken with villin (D) compared to control (C); cultivation with *L. casei* LOCK0919 for 7D stimulated villin to concentrate in narrow lines that may represent membrane structures of IEC-18 cells (F) compared to control (E).

B-Catenin is associated with adherens junctions (AJs) where it functions as a component of the cadherin complex that controls cell-cell adhesion by linking cadherin to the actin cytoskeleton (Nelson and Nusse 2004). The rate of β -catenin expression and localization was increased in the course of differentiation from cytoplasm on 3D (G) into cell membrane on 7D (I). *L. casei* LOCK0919 stimulated transfer of β -catenin to cell membrane of less differentiated cells on 3D to cell membrane (H) and intensified expression of β -catenin in cell membrane in more differentiated cells on 7D (J).

Discussion

This study was undertaken to examine whether the three probiotic *Lactobacillus* strains, known for anti-allergic response, can accelerate differentiation of non-transformed rat crypt-like small intestinal line IEC-18 and regulate its immune function including production of inflammatory cytokines. Nonmalignant IEC lines require for differentiation and return of cell polarization *in vitro* external basement membrane matrix or collagen gels supports to induce a spectrum of changes toward differentiation (Sanderson *et al.* 1996). Epithelial cells detect microbes through the Toll-like receptors as transmembrane signal transducing receptors. We showed that individual *Lactobacillus* strains are recognized differently by TLR-2 and TLR-4 of IEC-18 cells and that this interaction plays crucial role in acceleration of intestinal cell

differentiation from undifferentiated towards a more differentiated state. In this study, *L. rhamnosus* LOCK0908 was not modifying TLR-2 and TLR-4 mRNA expression in IEC-18 cells. Similarly, there is no interaction of *L. rhamnosus* LOCK0908 strain with HEK293 cells transfected with either TLR-2 or TLR-4, likely due to masking bioactive components for binding by its high level of surface exopolysaccharides (Kozakova *et al.* 2016). In contrast, *L. rhamnosus* LOCK0900 contain polysaccharides that in isolated form modulate immune response of *L. plantarum* WCFS1 by enhancing production of IL-10 and IL-12p70 of dendritic cells (Gorska *et al.* 2014). *L. rhamnosus* LOCK0900 is recognized by TLR-2 inducing its mRNA expression after IEC detachment from ECM and, in addition, also by inducing internal cytosolic NOD2 receptor by up-regulating its transcription. Stimulation of NOD2 leads to the activation of transcription factor NF- κ B (Martinon *et al.* 2004). *L. rhamnosus* LOCK0900 strain does not signal through TLR-4, consistent with HEK293/TLR-4 cell system (Kozakova *et al.* 2016).

L. casei LOCK0919 is recognized by both TLR-2 and TLR-4, as shown by up-regulation of their mRNA and increased immunostaining of their proteins in the cytoplasm. Similarly, *L. paracasei* NCC2461 acts through both TLR-2 and TLR-4 in HEK293 cell system (Schabussova *et al.* 2012). Our Western blot data show that the protein size of both TLR-2 and TLR-4 between 100-105 kDa after 7-d cultivation appeared to be partially glycosylated molecular forms with majority immunostaining in the cytoplasm of IEC-18 cells. Similarly, Ohnishi (Ohnishi *et al.* 2003) designed 110 kDa TLR-4 as partially glycosylated protein expressing TLR-4 without helper myeloid factor-2 (MD-2) on HEK293 cells. An even lesser glycosylated 97 kDa TLR-4 protein (Neal *et al.* 2006) and 85 kDa of TLR-2 and 92 kDa of TLR-4 (Cario *et al.* 2000) indicated both TLR-2 and TLR-4 with majority staining in the cytoplasm of IEC-6 cells. According to Ohnishi (Ohnishi *et al.* 2003) TLR-4 is able to undergo glycosylation at multiple positions, 110 kDa being partially glycosylated and the 130

kDa being the only one form expressed in a small portion on the cell surface. This specific glycosylation form of TLR-4 requires the presence of a helper myeloid factor-2 (MD-2) to function as a membrane receptor. Question arises by which mechanism bacterial translocation occurs across the intestinal barrier. It was shown that IEC can transfer bacteria into internalized TLR receptor vesicles or phagosomes upon the interaction of TLR with bacterial ligand (Neal *et al.* 2006, Sanderson and Walker 2007).

Recent tests demonstrate that *L. casei* LOCK0919 is a dominant strain colonizing mouse intestine due to high adherence to enterocytes originating from an unusual plasmid-localized pilus gene cluster (Aleksandrak-Piekarczyk *et al.* 2015). This is highly similar to that of *L. rhamnosus* GG and different from those of *L. rhamnosus* LOCK0900 and *L. rhamnosus* LOCK0908 which do not encode such pilus gene cluster. The increased adhesion of *L. casei* LOCK0919 to enterocytes may enhance protection against pathogen adhesion. Our results indicate that in the course of differentiation of intestinal epithelial cells *Lactobacillus* strains can directly stimulate inflammatory activities of IEC-18 cells including production of inflammatory cytokines of IL-1 family, IL-1 β and IL-18. These cytokines cause a variety of biological effects associated with infection and inflammation.

Cytokine IL-1 β is closely related to IL-18; both possess similar three dimensional structure and their precursor forms are inactive and remain intracellularly located until cleaved by cysteine protease caspase-1. However, there is a specific difference between IL-18 and IL-1 β . Whereas IL-18 is constitutively expressed in epithelial cells of the entire gastrointestinal tract, gene expression of IL-1 β in epithelial cells under normal conditions is absent (Dinarello *et al.* 2013) until stimulated by signals activating NF- κ B. IL-18, its receptors IL-18R α and IL-18R β and caspase-1 are constitutively expressed in rat IEC-6 line derived from the whole small intestine (Kolinska *et al.* 2008). Here we showed further that when activated at the apical surface of IEC-18 cells by a mixture of *L. casei* LOCK0919, *L.*

rhamnosus LOCK0900 and *L. rhamnosus* LOCK0908 the bacterially induced promoter sequence of IL-18 is induced, as proposed for lipopolysaccharide (LPS) by Foss (Foss *et al.* 2001).

Caspase-1 is constitutively activated within an inflammasome complex that includes intracellular receptors such as NLRP3, with structural similarity to NOD2 (Martinon *et al.* 2004) that activates conversion of pro-caspase-1 to active caspase-1 (Zaki *et al.* 2010). In IEC-18 extract we detected constitutive isoforms of active caspase-1, such as those reported before (Yamin *et al.* 1996, Feng *et al.* 2004) and stimulated by *L. casei* LOCK0919. Active caspase-1 is a prerequisite for obtaining biologically active mature and secreted IL-18 form. In this study, we showed that the polarized IEC-18 grown on collagen secreted IL-18 at both apical and basolateral poles depending on the state of differentiation. We speculate that *in vivo* at the basolateral side, IL-18 induces the production of chemokine IL-8 and facilitates inflammatory signaling through stimulation of IL-18R β (Kolinska *et al.* 2008) to induce IFN- γ and exert inflammatory properties. Conversely, at the apical pole and in the intestinal cells the secreted IL-18 mediates proliferation, protection and preservation of the intestinal barrier and restores healing of damaged cells during infection (Siegmund 2010).

Our results also demonstrate that *L. rhamnosus* LOCK0900 increases transcription of caspase-1 and that both *L. casei* LOCK0919 and *L. rhamnosus* LOCK0900 strains stimulate gene expression of the signal adaptor molecule MyD88. MyD88 is a functional molecule to activate NF- κ B critical for IL-18 and IL-1 β signaling (Adachi *et al.* 1998) via their receptors, as well as TLRs/MyD88 dependent production of inflammatory cytokines in infections (Seki *et al.* 2002). While IL-18 transcript is expressed as differentiation marker of IEC-18 cells cultivated on extracellular matrix and stimulated by *L. casei* LOCK0919, IL-1 β is not present in non-transformed IEC-18 cells (Dinarello *et al.* 2013).

It is known that IL-1 β gene expression is rapid as an early induced event of inflammation. Experimental procedure involving detachment of IEC-18 cells from extracellular matrix is considered as a stress stimulus for IL-1 β transcript production until 1 h after detachment (Waterhouse and Stadnyk 1999). In addition to stimulation of IL-1 β , we observed increased transcription of TLR-2 and IL-8 in detached cells in the presence of *L. rhamnosus* LOCK0900 strain. Previously was shown that IEC-6 cells respond to detachment by producing mRNA and protein of cytokine IL-6 (Miller and McGee 2002). Furthermore, detachment directly activates NF- κ B (Yan *et al.* 2005). We speculate that expressed TLR-2 mediates the signal from microbial components of *L. rhamnosus* LOCK0900 through MyD88 dependent pathway and stimulates transcription factor NF- κ B and IL-8 and IL-6 gene transcription. IL-6 gene expression may be also enhanced by IL-1 β (McGee *et al.* 1993). NF- κ B promotes expression of antiapoptotic and proapoptotic molecules (Yan *et al.* 2005). IL-1 β protects IEC from apoptosis, which may protect integrity of epithelial monolayer (Waterhouse *et al.* 2001). *L. rhamnosus* LOCK0900 also stimulates gene expression of cytosolic bacterial recognition molecule NOD2 in detached IEC-18 cells. This coincided with NOD2 gene expression in mouse ileal crypts dependent on commensal flora; *L. plantarum* increasing NOD2 mRNA in wild mice vs mRNA in germ-free mice (Petnicki-Ocwieja *et al.* 2009).

Cytoskeleton binding proteins villin and β -catenin were studied by immunofluorescence under the effect of *L. casei* LOCK0919 to evaluate the effect on differentiation of cellular morphology. This strain is known for strong adherence to enterocytes (Aleksandrak-Piekarczyk *et al.* 2015) and modulatory effect of its polysaccharides on immune response to *L. plantarum* WCFS1 (Gorska *et al.* 2016). We demonstrated that villin in IEC-18 cells is dependent on cell differentiation in ECM fashion. Villin was, however, detected in IEC-18 cells grown on glass as in Sanderson (Sanderson *et al.* 1996) and (Wang *et al.* 2003). We cultivated the cells on glass to get a better view of an

independent effect of bacteria. *L. casei* LOCK0919 accelerates subcellular distribution of villin, its localization from the cytoplasm approaching the cell membrane of a number of cells. B-catenin expression and change in localization were increased during IEC-18 cell differentiation. Its transfer from cytoplasm to cell membrane was stimulated by *L. casei* LOCK0919. In normal development, β -catenin functions in cell adhesion, linking catherin to α -catenin and binding this complex with the cytoskeleton (Harris and Peifer 2005). Free β -catenin plays a role in transcription via Wnt-canonical pathway regulating cell cycle, cell growth and proliferation. Inhibition of β -catenin signaling plays a key role in pathogenesis of necrotizing enterocolitis via impaired enterocyte proliferation (Sodhi *et al.* 2010).

In this study, we focused on the sequences of events in the process of differentiation of intestinal epithelial cells as an active participant in the mucosal response to the commensal *Lactobacillus* strains that can directly activate immune and morphological functions of intestinal epithelial cells. We support the notion that newly made membrane proteins are delivered to the surface with different speed, TLRs vs structural markers, and that degree of glycosylation is essential for surface expression. Different response of individual *Lactobacillus* strains to production of inflammatory cytokines points to tightly controlled specific induction of inflammatory cytokine IL-1 β in detached IEC-18 cells by *L. rhamnosus* LOCK0900.

Conflict of Interest

There is no conflict of interests.

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References

- ADACHI O, KAWAI T, TAKEDA K, MATSUMOTO M, TSUTSUI H, SAKAGAMI M, NAKANISHI K, AKIRA S: Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**: 143-150, 1998.
- ALEKSANDRZAK-PIEKARCZYK T, KORYSZEWSKA-BAGINSKA A, BARDOWSKI J: Genome Sequence of the Probiotic Strain Lactobacillus rhamnosus (Formerly Lactobacillus casei) LOCK900. *Genome Announc* **1**: 2013.
- ALEKSANDRZAK-PIEKARCZYK T, KORYSZEWSKA-BAGINSKA A, GRYNBERG M, NOWAK A, CUKROWSKA B, KOZAKOVA H, BARDOWSKI J: Genomic and Functional Characterization of the Unusual pLOCK 0919 Plasmid Harboring the spaCBA Pili Cluster in Lactobacillus casei LOCK 0919. *Genome Biol Evol* **8**: 202-217, 2015.
- ATHMAN R, LOUVARD D, ROBINE S: The epithelial cell cytoskeleton and intracellular trafficking. III. How is villin involved in the actin cytoskeleton dynamics in intestinal cells? *Am J Physiol Gastrointest Liver Physiol* **283**: G496-502, 2002.
- BARNICH N, AGUIRRE JE, REINECKER HC, XAVIER R, PODOLSKY DK: Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor- κ B activation in muramyl dipeptide recognition. *J Cell Biol* **170**: 21-26, 2005.
- BLOBEL G, WALTER P, CHANG CN, GOLDMAN BM, ERICKSON AH, LINGAPPA VR: Translocation of proteins across membranes: the signal hypothesis and beyond. *Symp Soc Exp Biol* **33**: 9-36, 1979.
- CARIO E, ROSENBERG IM, BRANDWEIN SL, BECK PL, REINECKER HC, PODOLSKY DK: Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol* **164**: 966-972, 2000.
- CUKROWSKA B, ROSIAK I, KLEWICKA E, MOTYL I, SCHWARZER M, LIBUDZISZ Z, KOZAKOVA H: Impact of heat-inactivated Lactobacillus casei and Lactobacillus paracasei strains on cytokine responses in whole blood cell cultures of children with atopic dermatitis. *Folia Microbiol (Praha)* **55**: 277-280, 2010.
- DINARELLO CA, NOVICK D, KIM S, KAPLANSKI G: Interleukin-18 and IL-18 binding protein. *Front Immunol* **4**: 289-299, 2013.
- FENG Q, LI P, LEUNG PC, AUERSPERG N: Caspase-1zeta, a new splice variant of the caspase-1 gene. *Genomics* **84**: 587-591, 2004.
- FOSS DL, ZILLIOX MJ, MURTAUGH MP: Bacterially induced activation of interleukin-18 in porcine intestinal mucosa. *Vet Immunol Immunopathol* **78**: 263-277, 2001.
- FUSUNYAN RD, NANTHAKUMAR NN, BALDEON ME, WALKER WA: Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes. *Pediatr Res* **49**: 589-593, 2001.
- GARDELLA S, ANDREI C, COSTIGLIOLO S, POGGI A, ZOCCHI MR, RUBARTELLI A: Interleukin-18 synthesis and secretion by dendritic cells are modulated by interaction with antigen-specific T cells. *J Leukoc Biol* **66**: 237-241, 1999.
- GOLIAS J, HUMLOVA Z, HALADA P, HABOVA V, JANATKOVA I, TUCKOVA L: Identification of rice proteins recognized by the IgE antibodies of patients with food allergies. *J Agric Food Chem* **61**: 8851-8860, 2013.
- GORSKA S, HERMANOVA P, CIEKOT J, SCHWARZER M, SRUTKOVA D, BRZOZOWSKA E, KOZAKOVA H, GAMIAN A: Chemical characterization and immunomodulatory properties of polysaccharides isolated from probiotic Lactobacillus casei LOCK 0919. *Glycobiology* **26**: 1014-1024, 2016.
- GORSKA S, SCHWARZER M, JACHYMEK W, SRUTKOVA D, BRZOZOWSKA E, KOZAKOVA H, GAMIAN A: Distinct immunomodulation of bone marrow-derived dendritic cell responses to Lactobacillus plantarum WCFS1 by two different polysaccharides isolated from Lactobacillus rhamnosus LOCK 0900. *Appl Environ Microbiol* **80**: 6506-6516, 2014.
- HARRIS TJ, PEIFER M: Decisions, decisions: beta-catenin chooses between adhesion and transcription. *Trends Cell Biol* **15**: 234-237, 2005.

- HEATH JP: Epithelial cell migration in the intestine. *Cell Biol Int* **20**: 139-146, 1996.
- ISMAIL AS, HOOPER LV: Epithelial cells and their neighbors. IV. Bacterial contributions to intestinal epithelial barrier integrity. *Am J Physiol Gastrointest Liver Physiol* **289**: G779-784, 2005.
- JALAL F, JUMARIE C, BAWAB W, CORBEIL D, MALO C, BERTELOOT A, CRINE P: Polarized distribution of neutral endopeptidase 24.11 at the cell surface of cultured human intestinal epithelial Caco-2 cells. *Biochem J* **288 (Pt 3)**: 945-951, 1992.
- KEDINGER M: What triggers intestinal cells to move or stay and to proliferate or differentiate? *Gastroenterology* **107**: 885-888, 1994.
- KOLINSKA J, LISA V, CLARK JA, KOZAKOVA H, ZAKOSTELECKA M, KHAILOVA L, SINKORA M, KITANOVICOVA A, DVORAK B: Constitutive expression of IL-18 and IL-18R in differentiated IEC-6 cells: effect of TNF-alpha and IFN-gamma treatment. *J Interferon Cytokine Res* **28**: 287-296, 2008.
- KORYSZEWSKA-BAGINSKA A, ALEKSANDRZAK-PIEKARCZYK T, BARDOWSKI J: Complete Genome Sequence of the Probiotic Strain Lactobacillus casei (Formerly Lactobacillus paracasei) LOCK919. *Genome Announc* **1**: 2013.
- KORYSZEWSKA-BAGINSKA A, BARDOWSKI J, ALEKSANDRZAK-PIEKARCZYK T: Genome Sequence of the Probiotic Strain Lactobacillus rhamnosus (Formerly Lactobacillus casei) LOCK908. *Genome Announc* **2**: 2014.
- KOZAKOVA H, SCHWARZER M, TUCKOVA L, SRUTKOVA D, CZARNOWSKA E, ROSIAK I, HUDCOVIC T, SCHABUSSOVA I, HERMANOVA P, ZAKOSTELSKA Z, ALEKSANDRZAK-PIEKARCZYK T, KORYSZEWSKA-BAGINSKA A, TLASKALOVA-HOGENOVA H, CUKROWSKA B: Colonization of germ-free mice with a mixture of three lactobacillus strains enhances the integrity of gut mucosa and ameliorates allergic sensitization. *Cell Mol Immunol* **13**: 251-262, 2016.
- MARTINON F, AGOSTINI L, MEYLAN E, TSCHOPP J: Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. *Curr Biol* **14**: 1929-1934, 2004.
- MCGEE DW, BEAGLEY KW, AICHER WK, MCGHEE JR: Transforming growth factor-beta and IL-1 beta act in synergy to enhance IL-6 secretion by the intestinal epithelial cell line, IEC-6. *J Immunol* **151**: 970-978, 1993.
- MICHELSSEN KS, ARDITI M: Toll-like receptors and innate immunity in gut homeostasis and pathology. *Curr Opin Hematol* **14**: 48-54, 2007.
- MILLER TL, MCGEE DW: Epithelial cells respond to proteolytic and non-proteolytic detachment by enhancing interleukin-6 responses. *Immunology* **105**: 101-110, 2002.
- NEAL MD, LEAPHART C, LEVY R, PRINCE J, BILLIAR TR, WATKINS S, LI J, CETIN S, FORD H, SCHREIBER A, HACKAM DJ: Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. *J Immunol* **176**: 3070-3079, 2006.
- NELSON WJ, NUSSE R: Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**: 1483-1487, 2004.
- OHNISHI T, MUROI M, TANAMOTO K: MD-2 is necessary for the toll-like receptor 4 protein to undergo glycosylation essential for its translocation to the cell surface. *Clin Diagn Lab Immunol* **10**: 405-410, 2003.
- PETNICKI-OCWIEJA T, HRNCIR T, LIU YJ, BISWAS A, HUDCOVIC T, TLASKALOVA-HOGENOVA H, KOBAYASHI KS: Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci U S A* **106**: 15813-15818, 2009.
- RUBARTELLI A, COZZOLINO F, TALIO M, SITIA R: A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J* **9**: 1503-1510, 1990.
- SANDERSON IR, EZZELL RM, KEDINGER M, ERLANGER M, XU ZX, PRINGAULT E, LEON-ROBINE S, LOUVARD D, WALKER WA: Human fetal enterocytes in vitro: modulation of the phenotype by extracellular matrix. *Proc Natl Acad Sci U S A* **93**: 7717-7722, 1996.
- SANDERSON IR, WALKER WA: TLRs in the Gut I. The role of TLRs/Nods in intestinal development and homeostasis. *Am J Physiol Gastrointest Liver Physiol* **292**: G6-10, 2007.
- SEKI E, TSUTSUI H, TSUJI NM, HAYASHI N, ADACHI K, NAKANO H, FUTATSUGI-YUMIKURA S, TAKEUCHI O, HOSHINO K, AKIRA S, FUJIMOTO J, NAKANISHI K: Critical roles of myeloid differentiation

- factor 88-dependent proinflammatory cytokine release in early phase clearance of *Listeria monocytogenes* in mice. *J Immunol* **169**: 3863-3868, 2002.
- SCHABUSSOVA I, HUFNAGL K, TANG ML, HOFLEHNER E, WAGNER A, LOUPAL G, NUTTEN S, ZUERCHER A, MERCENIER A, WIEDERMANN U: Perinatal maternal administration of *Lactobacillus paracasei* NCC 2461 prevents allergic inflammation in a mouse model of birch pollen allergy. *PLoS One* **7**: e40271, 2012.
- SIEGMUND B: Interleukin-18 in intestinal inflammation: friend and foe? *Immunity* **32**: 300-302, 2010.
- SODHI CP, SHI XH, RICHARDSON WM, GRANT ZS, SHAPIRO RA, PRINDLE T, JR., BRANCA M, RUSSO A, GRIBAR SC, MA C, HACKAM DJ: Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. *Gastroenterology* **138**: 185-196, 2010.
- STADNYK AW, JOSEPH RR, BLAKE KM: Regulation of cytokine expression in intestinal epithelial cells. *Mod Asp Immunol* **2**: 73-77, 2001.
- VALLEE S, LAFOREST S, FOUCHIER F, MONTERO MP, PENEL C, CHAMPION S: Cytokine-induced upregulation of NF-kappaB, IL-8, and ICAM-1 is dependent on colonic cell polarity: implication for PKCdelta. *Exp Cell Res* **297**: 165-185, 2004.
- WANG Z, CHEN WW, LI RL, WEN B, SUN JB: Effect of gastrin on differentiation of rat intestinal epithelial cells in vitro. *World J Gastroenterol* **9**: 1786-1790, 2003.
- WATERHOUSE CC, JOSEPH RR, STADNYK AW: Endogenous IL-1 and type II IL-1 receptor expression modulate anoikis in intestinal epithelial cells. *Exp Cell Res* **269**: 109-116, 2001.
- WATERHOUSE CC, STADNYK AW: Rapid expression of IL-1beta by intestinal epithelial cells in vitro. *Cell Immunol* **193**: 1-8, 1999.
- YAMIN TT, AYALA JM, MILLER DK: Activation of the native 45-kDa precursor form of interleukin-1-converting enzyme. *J Biol Chem* **271**: 13273-13282, 1996.
- YAN SR, JOSEPH RR, ROSEN K, REGINATO MJ, JACKSON A, ALLAIRE N, BRUGGE JS, JOBIN C, STADNYK AW: Activation of NF-kappaB following detachment delays apoptosis in intestinal epithelial cells. *Oncogene* **24**: 6482-6491, 2005.
- ZAKI MH, BOYD KL, VOGEL P, KASTAN MB, LAMKANFI M, KANNEGANTI TD: The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* **32**: 379-391, 2010.

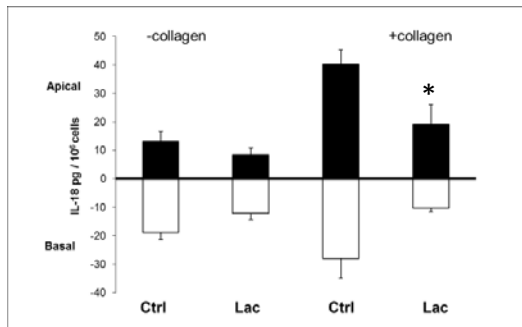
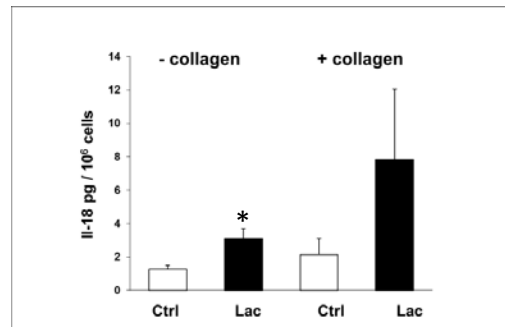
A**B**

Fig. 1. Interaction between IEC-18 line and ECM is important for cell differentiation. **(A)** Polarity of IL-18 secretion by IEC-18 cells. The cells are a source of IL-18 and secrete this cytokine from both their apical and basolateral poles. In response to apical probiotic equal-part mixture *L. rhamnosus/casei* (Lac) the IL-18 secretion is depressed but stays polar. **(B)** Lac enhances IL-18 protein accumulation in IEC-18 cell layer. In the group with collagen IL-18 production in IEC-18 cells was not significant for higher scatter of data. All numerical data (IL-18 pg/10⁶ cells assayed by ELISA) are expressed as mean \pm SEM of three groups of wells (4 transwells/group) for each point. *P<0.05, Lac vs control.

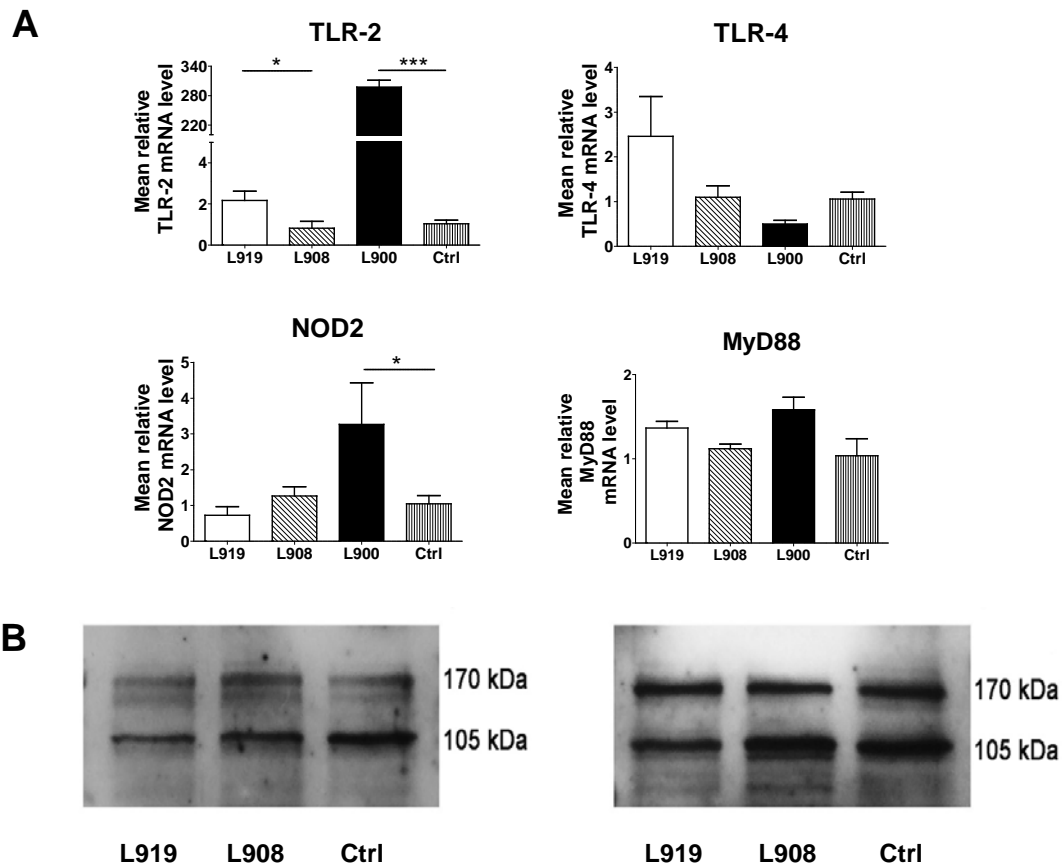


Fig. 2. Both TLR-2 and TLR-4 mRNA (**A**) and protein (**B**) are expressed constitutively in IEC-18 cell lysates after 7-day cultivation on Matrigel in untreated IEC. (**A**) *L. rhamnosus* LOCK0900 (L900) (10^7 CFU/ml) exposure for 2 h stimulated expression of TLR-2 mRNA and NOD2 mRNA. The stimulating effect of *L. casei* LOCK0919 (L919) on mRNA TLR-2 and insignificant stimulation of mRNA TLR-4 for higher scatter of data were observed. Stimulation of adapter molecule MyD88 mRNA by *L. rhamnosus* LOCK0900 (L900) and *L. casei* LOCK0919 (L919) was found insignificant. *L. rhamnosus* LOCK0908 (L908) exposure to IEC did not differ from control values. (**B**) Representative Western blot with goat anti TLR-2 and goat anti TLR-4 followed with peroxidase-labeled donkey IgG antibody revealed expression of 100-105 kDa protein bands of TLR-2 and TLR-4 in control as well as under the effect of *L. casei* LOCK0919 (L919) or *L. rhamnosus* LOCK0908 (L908). In (A) average mean \pm SEM of three experiments are presented. * $P < 0.05$, L919 vs control, *** $P < 0.001$, L900 vs control

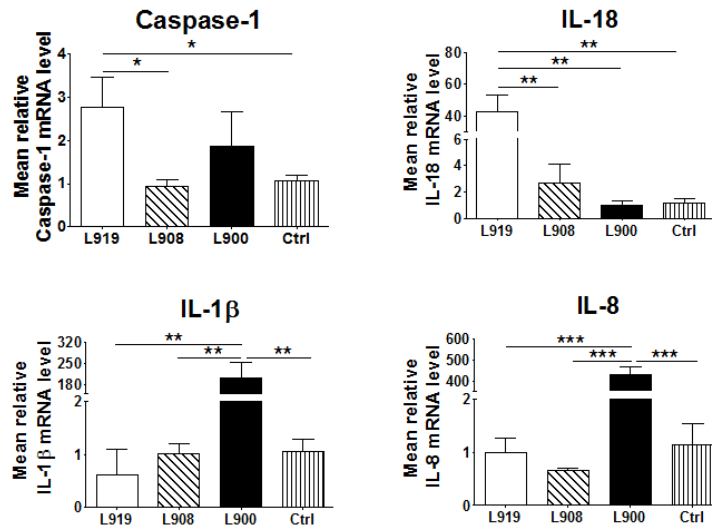
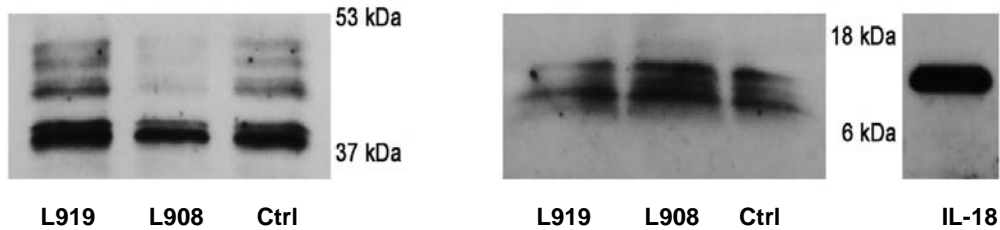
A**B**

Fig. 3. Caspase-1, IL-18, IL-1 β and IL-8 mRNAs expression in IEC-18 cells is differently affected by *L. rhamnosus* LOCK0900 (L900), *L. rhamnosus* LOCK0908 (L908) and *L. casei* LOCK09019 (L919) strains. (A) Mean relative caspase-1 mRNA level and mean relative IL-18 mRNA level are stimulated in the presence of *L. casei* LOCK0919 (L919) vs control, * $P < 0.05$ and ** $P < 0.01$, respectively. On the other hand, strain *L. rhamnosus* LOCK0900 (L900) induced mean relative mRNA levels of IL-1 β and IL-8. (B) Western blot analysis showed that cultured IEC-18 cells contain active mature caspase-1 (six protein bands between 37-53 kDa) in both control and that elevated by *L. casei* LOCK0919 (L919). Western blot showed three bands of IL-18 among 6-18 kDa correlated with the position of recombinant rat IL-18. Average mean \pm SEM of three experiments are presented. * $P < 0.05$, L919 vs control, ** $P < 0.01$, L919 and L900 vs control, *** $P < 0.001$, L900 vs control.

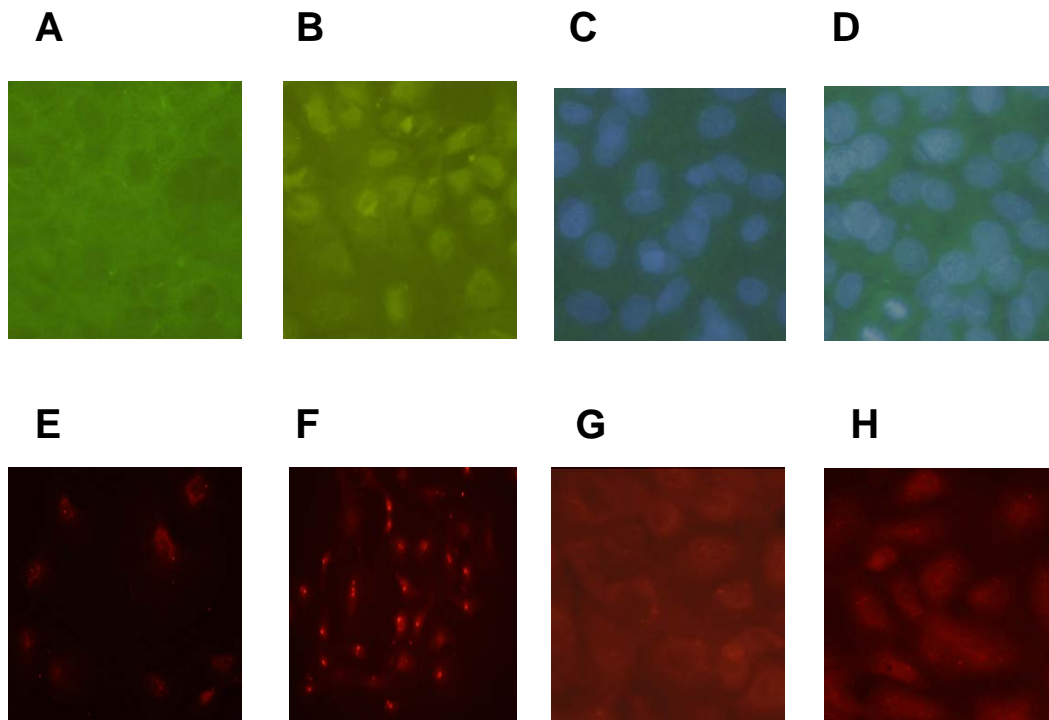


Fig. 4. Immunofluorescent staining of TLR-2, TLR-4, caspase-1 and IL-18 on 7D IEC-18 cultures at a magnification of 40 x. Images were taken in the center of the wells. TLR-2 in (A, B) and TLR-4 in (C, D) were stained green in cytoplasm. Nuclei visualized with DAPI (blue in C, D) showed even distribution of cells. IL-18 in (E, F) and caspase-1 in (G, H) were stained red in cytoplasm. *L. casei* LOCK0919 (L919) present 2 hours in culture stimulated expression of TLR-2, TLR-4, IL-18 and caspase-1 in (B, D, F, H) compared to controls in (A, C, E, G). Staining procedure with primary and secondary antibodies as presented in Materials and Methods.

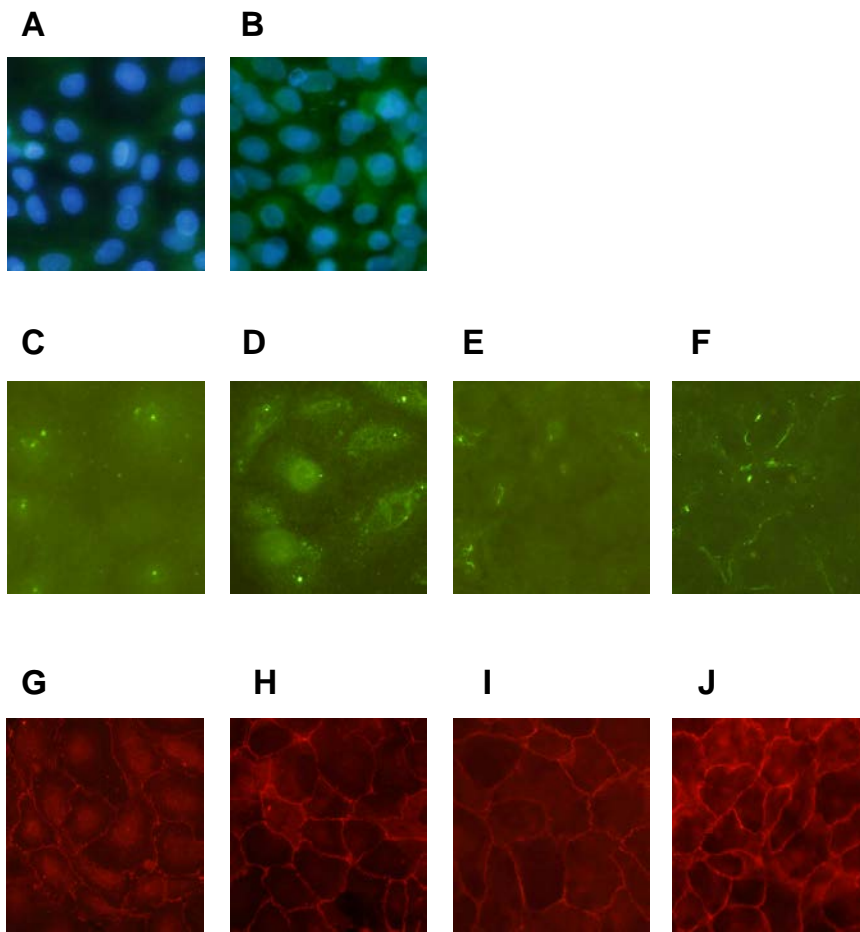


Fig. 5. Villin and β -catenin expression in control and in *L. casei* LOCK0919 (L919) treated IEC-18 culture evaluated by fluorescent microscopy; villin (green), β -catenin (red). (A) IEC-18 5D control culture, villin expressed near the nuclei (DAPI, blue). (B) In IEC-18 5D culture on Matrigel villin expression was stimulated in cytoplasm. (C) Control culture on 3D and (E) control culture on 7D, villin evenly distributed in cytoplasm. *L. casei* LOCK0919 stimulated expression of villin in the cytoplasm of 3D culture (D) and in 7D culture (F) with villin expression appearing in membrane structures of some cells. (G) In 3D control culture β -catenin is expressed mainly in cytoplasm, while in (H) under the effect of *L. casei* LOCK0919 transfer to cell membrane is seen. In 7D control culture most of β -catenin is expressed on cell membranes (I). Cultivation with *L. casei* LOCK0919 (J) increased β -catenin expression in the cytoplasm of some cells and intensified β -catenin association with membranes.