Szent István University

Doctoral School of Veterinary Science

Strategies for protecting enterocytes from oxidative stress-induced inflammation

PhD thesis

Pásztiné Dr. Gere Erzsébet

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Szent István University

Doctoral School of Veterinary Science

Supervisor and members of the project committee:

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Prof. Dr. Péter Gálfi DSc

Supervisor, Full Professor

Szent Istvan University, Faculty of Veterinary Sciences

Department of Pharmacology and Toxicology

Krisztina Szekér PhD

Member of the Project Committee

Szent Istvan University, Faculty of Veterinary Sciences

Department of Pharmacology and Toxicology

Csibrik-Németh Edina PhD

Member of the Project Committee

Szent Istvan University, Faculty of Veterinary Sciences

Department of Pharmacology and Toxicology

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Pásztiné dr Gere Erzsébet

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Abbreviations

4-HNE: 4-hydroxy-2-nonenal
AA: acetic acid
B. amyloliquefaciens: *Bacillus amyloliquefaciens* CECT 5940
BB-12: *Bifidobacterium animalis* subsp. *lactis* Bb 12
Caco-2 cell line: human colon adenocarcinoma cell line
cc-PE insert: collagen-coated polyester insert
CDs: conjugated dienes
CFU: colony-forming unit
CTs: conjugated trienes
DAPI: 4',6-diamidino-2 phenylindole
DSS: dextran sodium sulfate
E. faecium: *Enterococcus faecium* CECT 4515
FMOC: fluorenylmethyloxycarbonyl chloride
FOS: fructo-oligosaccharides
GALT: gut-associated lymphoid tissue
GOS: galacto-oligosaccharides
GPx: glutathione peroxidase
HRP: horseradish peroxidase
Hsp70: heat shock protein 70
IEC: intestinal epithelial cell
IL-6: interleukin-6   IL-8: interleukin-8
L. plantarum 2142: *Lactobacillus plantarum* 2142
LA: lactic acid
MALT: mucosa-associated lymphoid tissue
MDA: malondialdehyde
MRS: DeMan, Rogosa, Sharpe broth
NSAIDs: non-steroidal anti-inflammatory drugs
NRU: neutral red uptake assay
o-DA: o-dianisidine
OPA: o-phthalaldehyde
PBS: phosphate buffered saline
PC insert: polycarbonate insert
PKC: protein kinase C
PUFA: polyunsaturated fatty acids
PRR: pattern recognition receptor
ROS: reactive oxygen species
SB: sodium butyrate
SCFAs: short-chain fatty acids
SCS: spent culture supernatant
TB: trypan blue exclusion assay
TCA: trichloroacetic acid
TER: transepithelial electrical resistance
TJ: tight junction
TNF-α: tumor necrosis factor $-\alpha$
TPY: tryptone phytone yeast
TSB: tryptone soya broth
XOS: xylo-oligosaccharides

Summary

Probiotics have proven beneficial effects in the treatment of several intestinal infections, but the underlying mechanisms of how they can affect responses of porcine enterocytes to oxidative stress has not been fully revealed. It has previously been reported that probiotics can improve intestinal microbial balance, confer protection against potential enteropathogenic bacteria, and prevent or cure intestinal diseases. These effects are mediated via the production of antimicrobial metabolites such as salts of various short-chain carboxylic acids (lactate, acetate, propionate and butyrate), hydrogen peroxide or bacteriocins, and competition with harmful bacteria for nutrients or adhesion sites.

The aim of this study was to investigate the subcellular effects of acute oxidative stress using *in vitro* systems such as human colon adenocarcinoma cell line Caco-2 and non-transformed porcine intestinal epithelial IPEC-J2 cells. The sensitivities of these cell lines to oxidative stimuli were compared to each other based on transepithelial electrical resistance values and DAPI-indicated cell death. Optimization of the cell culturing conditions to maintain higher polarization rate of the cells was accomplished using different membrane inserts (collagen-coated polyester and polycarbonate type) for 3D models. Oxidative stress was induced by individually tailored peroxide treatment based on previous estimation of the dose dependencies and post-treatment time course of H_2O_2-induced cytokine expression. The extent of cell death was monitored using three different staining methods such as DAPI, neutral red uptake assay and trypan blue exclusion. Investigation of pro-inflammatory cytokine profile (IL-8 and TNF-α) and cytoprotective activity (Hsp 70) based on relative gene expression determination by qRT-PCR method was also performed. In addition, at the level of protein expression quantitative analysis of IL-8 in apical and basolateral compartments was carried out using ELISA. The peroxide-triggered cell response profile was evaluated by measuring TER change as an indicator of cell monolayer integrity and by monitoring formation of LPO byproducts such as early markers, conjugated dienes, conjugated trienes besides malondialdehyde present in later stage of peroxidation processes. To test the hypothesis whether paracellular gate opening occurs in IPEC-J2 cells exposed to 1 h peroxide treatment gentamicin transport study was conducted. In order to determine if millimolar peroxide could trigger redistribution of tight junctional proteins, immunohistochemical staining of claudin-1, claudin-4 and claudin-7 and E-cadherin was performed in IPEC-J2 cells and in small intestinal samples of unsuckled newborn and adult swines. This work involved Western blot analysis of the PKC isoenzyme pattern to reveal which isoenzyme if elevated

might be responsible for oxidative stress induced changes at signal transduction level during the recovery period after H_2O_2 administration. In the second part of the study, immunmodulatory effects of spent culture supernatants (SCSs) from five bacterial strains (*Lactobacillus plantarum* 2142, *Lactobacillus casei* Shirota, *Bifidobacterium animalis* subsp. *lactis* BB-12, *Bacillus amyloliquefaciens* CECT 5940 *Enterococcus faecium* CECT 4515) on the upregulation of IL-8 and TNF-α level were investigated by qRT-PCR. Dianisidine-based spectrophotometry was used for the quantitative determination of spontaneous decomposition of hydrogen peroxide and for estimation of chemical interaction between SCSs of probiotics and hydrogen peroxide. In addition, our goal was also to identify the active components (short-chain carboxylic acids such as acetic acid, lactic acid, butyric acid or peptide derivatives) among metabolites in SCS that can play a major role in this beneficial effect.

TER values of Caco-2 cells are highly variable compared to those measured with IPEC-J2 cells when both were cultured on membrane inserts, suggesting a lack of homogeneity in case of Caco-2 cells with respect to differentiation rate. Remarkable difference in reactivity towards oxidative stress was seen when comparing these two cell lines: IPEC-J2 cell monolayer integrity can be partially disrupted by a 1 h treatment with 2 mM hydrogen peroxide in contrast to Caco-2P cells, where more than 10 mM H_2O_2 is needed to achieve the same TER-decreasing effect in this treatment period. The higher sensitivity of IPEC-J2 cells to oxidative stimuli makes the 3D model capable of detecting physiological and redox cellular changes when jejunal epithelium is exposed to inflammatory processes of oxidative origin. We developed a H_2O_2 treatment regimen with optimized incubation time and H_2O_2 dose for achieving the peak level of IL-8 and TNF-α cytokine secretion without detectable cell death. This is the first study in which a porcine non-tumorigenic intestinal epithelial cell line was exposed to H_2O_2 alone and in combination with probiotics and changes in TER and relative gene expression levels of proinflammatory cytokines (IL-8 and TNF-α) and Hsp 70 were monitored with concomitant characterization of the anti-inflammatory *L. plantarum* 2142 supernatant. It was proven that 1 mM H_2O_2 treatment for 1 h led to significant upregulation of the proinflammatory cytokines, but at the same time this treatment did not affect the cellular localization of the investigated tight junctional proteins, claudin-1, claudin-4, claudin-7 and E-cadherin and the rate of lipid peroxidation based on the results of CDs, CTs and MDA measurements. We found that SCS of *Lactobacillus plantarum* 2142 at the concentration of 13.3% could effectively alleviate inflammatory processes formed as a consequence of excessive oxidative stress via restoration of upregulated IL-8 and TNF-α relative gene expression and via elevation of levels of cytoprotective Hsp70.

Our experiments also showed that the immunmodulatory effect of SCS was not based on its peroxide-decomposing activity due to the assumed scavenging properties, since the peroxide amount did not change in the presence of SCS apart from SCS of *Bacillus amyloliquefaciens*.

The fact that *L. plantarum* 2142 SCS alone could decrease the production of proinflammatory cytokines underlines the importance of active bacterial metabolites acting as efficient quenchers of oxidative stress-induced acute inflammatory responses. The major component in the spent culture supernatant of selected probiotics is lactic acid (LA). D- and L-lactic acid content was determined by enantiomer-selective lactate dehydrogenase-based kit. *L. plantarum* 2142, which produced the highest amount of D- and L-lactic acid showed beneficial effect in quenching oxidative stress induced inflammation to the greatest extent. This raised the question whether the lactic acid present in the supernatant is responsible for the anti-inflammatory properties exerted by *lactobacilli*. We found that none of the secreted lactic acid enantiomers was capable of preventing IECs from injury when cells were exposed to acute oxidative stress. The putative role of another metabolite, acetic acid (AA) in peroxide-triggered acute oxidative stress was also investigated via elucidation of changes in IL-8 and TNF-α relative gene expression. No beneficial effect of acetic acid was detected when cells were treated with 1 mM hydrogen peroxide. Protein analysis with SDS-PAGE and capillary zone electrophoresis revealed the presence of peptides of different molecular weights in the SCSs of *Lactobacillus plantarum* 2142, which components might play an active role in the suppression of upregulated proinflammatory cytokine levels and contribute to the cytoprotective activity through elevation of Hsp 70 level.

Based on our results we concluded that butyrate exerts its anti-inflammatory effects through improvement of the barrier function of oxidative stress-affected gastrointestinal epithelium, facilitation of enterocyte proliferation in normal intestinal tissue and maintenance of healthy gut microbiota and lactobacilli-enriched acidic milieu *in vivo*. The suppression of pathogenicic *E. coli* 30037 growth could be observed *in vitro* when butyrate was added at 11 mM concentration to the bacterial media, which, on the other hand, did not affect the number of *lactobacilli* under culturing conditions.

Probiotics such as *Lactobacillus casei* Shirota, *Bifidobacterium animalis* subsp. *lactis* BB-12, *Enterococcus faecium* CECT 4515 did not decrease the peroxide-induced changes in IL-8 and TNF-α relative gene expression. In addition, *Bacillus amyloliquefaciens* CECT 5940 further potentiated the oxidative stress-induced upregulation of proinflammatory cytokine level.

1. Introduction and literature overview

1.1 Introduction

Intestinal epithelium acts as a strong physical and chemical barrier against invading bacteria, toxins, oxidative stress and various chemical agents. Malfunction of the epithelial defense mechanisms as a result of damaged gut mucosa and altered intestinal microbial homeostasis can easily lead to leaky gut syndrome which can influence the general health condition even of animals on optimal nutritional regimens.

Reactive oxygen species (ROS) can easily damage proteins thereby accelerating the turnover of peptides with concomitant decrease in enzyme activity and at the same time lipid peroxidation can take place putting physiological cellular membrane function at risk. Late phase lipid peroxidation byproducts, such as aldehydes may cause severe DNA damage leading to mutation and alterations in carbohydrate metabolism. Free radicals have been often considered as triggering factors in the development of acute and chronic intestinal inflammation accompanied by cell and tissue injury and epithelial barrier disruption via tight junction (TJ) protein disassembly in apical junctional complexes.

Low-dose dietary antibiotics, previously used as growth promoters had been widely used into livestock production. The advantages of low-dose antibiotics include improvement in average daily weight gain and feed efficiency. However, since 2006 the use of low-dose dietary antibiotics has been banned in the European Union in connection with the growth promotion of livestock to avoid development of antibiotic resistance of some pathogenic bacteria. Due to the strict regulation on application of low-dose antibiotics for growth promotion, monogastric animal feed industry could only compete with production of animal feed supplemented with probiotics against countries outside the EU still using in-feed antimicrobials for preventing animals from diseases such as scour and necrotic enteritis. Probiotics are defined as live microbial food/feed ingredients that have a beneficial effect on the host health and well-being. They are normal inhabitants of the healthy gut microbiota and present in several fermented foods such as cheese and milk. Recently, probiotics represent one of the most promising alternatives to antibiotics to protect animal health and increase the efficiency of nutrient utilization.

Fatty acids are widely used to reduce mucosal damage caused by infection or oxidative stress in swine. Among the most important fatty acids are SCFA, particularly butyrate, produced by intestinal microbiota (mostly probiotic bacteria) can play important role

in the physiology and metabolism of the rumen, the intestine and the ruminal and intestinal mucosa. In addition to serving as a preferred energy source for colonocytes, butyrate has been implicated in protection against colon cancer and ulcerative colitis. Butyrate is produced by intestinal bacteria from prebiotic carbohydrates such as resistant starch, dietary fiber, inulin and fructo-oligosaccharides (FOS) that escape digestion in the small intestine. Augmentation of butyrate production in the intestine would be desirable for the maintenance of colonic health in both humans and animals.

Oxidative stress via causing cell and tissue damage can lead to formation of acute and chronic inflammation. The goal of our experimental work was the development of an *in vitro* system mimicking intestinal epithelium, where oxidative stimuli can be introduced by peroxide treatment and the pathophysilogical effect of acute oxidative stress can be monitored continuously. The prerequisite for finding the optimal dose and treatment time of peroxide administration was the maintenance of cell viability whereas the changes in relative gene expression level of pro-inflammatory cytokines could indicate the acute phase of inflammatory processes. In addition, the aim of this study was to assess the influence of spent culture supernatant (SCS) of potential probiotics (*Lactobacillus plantarum* 2142, *Lactobacillus casei* Shirota, *Bifidobacterium animalis* subsp. *lactis* BB-12, *Bacillus amyloliquefaciens* CECT 5940 *Enterococcus faecium* CECT 4515) on the response of enterocytes to oxidative stress, and the ability of SCS to protect from oxidative injury (**Fig. 1)** and to find out which components of the SCS are responsible for this beneficial effect.

To determine the impact of probiotics on acute oxidative stress-induced inflammation, experiments were performed employing IPEC-J2 intestinal epithelial cell line (cultured on collagen-coated polyester membrane inserts), cell line of non-transformed enterocytes isolated from the jejunum of a neonatal piglet. In veterinary studies IPEC-J2 cells have been used to investigate the interactions of various enteric pathogens [Brosnahan et al 2012, Skjolaas et al 2006, Brown et al 2007] including *Salmonella enterica* and pathogenic *Escherichia coli*. Enterotoxigenic *Escherichia coli* (ETEC) infections result in large economic losses in the swine industry worldwide proving usefulness of non-transformed porcine intestinal cell lines for studying ETEC pathogenesis [Koh et al 2008]. IPEC-J2 cell line also seems to be a reliable model for *in vitro* assessment of antibiotics' intestinal absorption in animals exposed to *Fusarium* mycotoxins deoxynivalenol and T-2 toxin [Goossens et al 2012].

We used hydrogen peroxide solution to provoke oxidative stress. and we determined via qRT- PCR method the relative gene expression of two inflammatory cytokines (IL-8 and TNF-α)

and that of cytoprotective 70 kDa heat shock protein (Hsp70) affected by SCS or short-chain carboxylic acids.
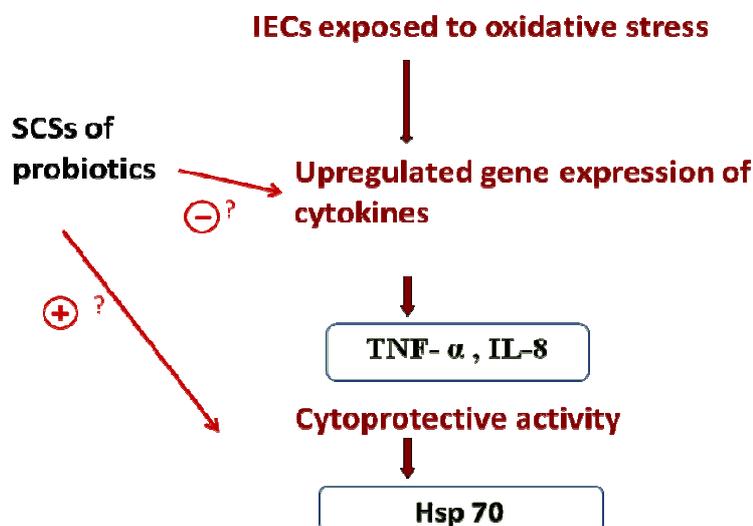
Fig. 1 Pathway analysis of excessive oxidative stress-modified cytokine profile and potential strategies to prevent IPEC-J2 cells from oxidative injury by candidate compounds. Spent culture supernatants of different probiotics were applied to trace their potential attenuating effect on upregulation of proinflammatory cytokines and their stimulatory effect on cytoprotective activity. IECs: intestinal epithelial cells SCS: spent culture supernatant

1.2. Intestinal microbiota as gatekeeper of healthy gut

The gut-associated microbes colonize superficial body sites such as skin, the airways and gastrointestinal tract. The intestine seems to be an important target in the prevention of allergic conditions such as asthma, eczema, rhinitis and food allergies driven by disregulated immune responses toward antigens. The role of microbe-host interactions in allergic diseases has been extensively studied: Many different bacterial strains and their mixture, synbiotics (combination of prebiotics and probiotics) were used as part of targeted therapy in clinical trials focused on allergic sensitization without conclusive results [Kalliomaki et al 2003, Dotterud et al 2010, Gruber et al 2007]. Thus, more studies are needed to pre-select bacterial strains with a high protective potential and to unravel the underlying mechanisms of altered microbe-host interaction in allergy development [Hörmannsperger et al 2012].

Genetic predisposition and environmental factors (**Fig. 2**) can act as key regulators in promoting the development of autoimmune diseases such as multiple sclerosis. Activation of B cells takes place in the germinal centres of the lymph nodes. The activated cells produce

antibodies against the myelin layer in the brain thus contributing to the occurence of inflammatory responses. It has not been revealed which bacteria are involved in the formation of multiple sclerosis. Analysis of the microbial genome can point out the differences between intestinal microbiota of healthy individuals and multiple sclerosis patients [Berer 2011].
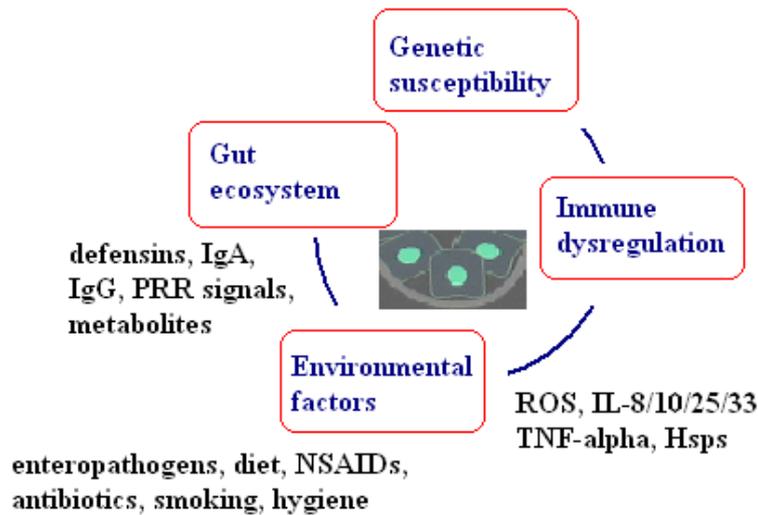
Fig. 2 Coordinated interplay between external and internal factors behind inflammation
PRR: pattern recognition receptor, ROS: reactive oxygen species, Hsp: heat shock protein,
NSAID: non-steroidal anti-inflammatory drugs

The colocalization of host and microbes involves a variety of molecular mechanisms, which contribute to dynamic and peaceful interaction between commensal bacterial species and the intestinal epitelium in living organisms. However, this delicate balance can be easily tipped by deterioration in intestinal microbiota due to the changes in bacterial species abundance and diversity. It has not been fully understood yet, whether the altered commensal bacterial profile is the cause or consequence of development of immune-mediated chronic gastrointestinal diseases such as idiopathic pathologies ulcerative colitis and Crohn's disease. In the treatment of inflammatory bowel diseases the main task is to restore the modified intestinal homeostasis via reestablishment of host-microbial relationship [Haller et al 2012].

Dendritic cells (DCs) have a pivotal role in the dialogue between host immune system and exogeneous stimuli and activation of T cell-mediated immune responses. In inflammatory bowel syndrome DCs are activated, expression of microbial recognition receptors is elevated with upregulated cytokine production (IL-6 and IL-12) makes them putative candidates for initiation of inflammatory responses in Crohn's disease [Hart et al 2005]. Probiotics can also

facilitate maturation of DCs and they are potential activators of T cells [Foligne et al 2007, Hart et al 2004].

There is emerging evidence that the application of probiotics can represent a novel science-based approach in the prevention of metabolic syndrome characterized as predisposing condition leading to cardiovascular disorder or diabetes mellitus with insulin resistance [Cencic et al 2012]. By the aid of genetically modified *Lactococcus lactis*, a common and food-grade commensal non-pathogenicic bacterium autoimmune diabetes type 1 characterized by breach in tolerance toward pancreatic insulin-producing β cells could be reversed. This novel approach appeared to work as effective treatment strategy for autoimmune diabetes by tolerance restoration using mucosal delivery of lactococci designed to secrete proinsulin autoantigen along with the immunmodulatory cytokine, IL-10 in mice [Takiishi 2012].

1.3. Mucosal immune response

The pig has a digestive system which is classified as monogastric or nonruminant. The small intestine with three anatomical segments, the duodenum, the jejunum and the ileum. It is the largest component of the digestive tract and the major site of digestion and absorption. The epithelium of the small intestine consists of six different cell types, namely enterocytes, goblet cells, Paneth cells, enteroendocrine cells, M cells and stem cells.

Mucosa-associated lymphoid tissue (MALT) includes gut-associated lymphoid tissue (GALT), bronchial/tracheal-associated lymphoid tissue (BALT), nose-associated lymphoid tissue (NALT), and vulvovaginal-associated lymphoid tissue (VALT). Additional mucosa-associated lymphoid tissue (MALT) exists within the accessory organs of the digestive tract. The gastrointestinal immune system is comprised of the lymphoid tissues collectively referred to as the gut-associated lymphoid tissue or GALT. The number of lymphocytes in the GALT is roughly equal to those in the spleen. Peyer's patches are lymphoid follicles similar in many ways to lymph nodes, located in the mucosa and [Featherstone 1997, Hamzaoui and Pringault 1998] extending into the submucosa of the small intestine, especially the ileum. In adults, B lymphocytes predominate in Peyer's patches. Smaller lymphoid nodules can be found throughout the intestinal tract. Lymphocytes can also be found in the basolateral spaces between luminal epithelial cells in the epithelium. The other part of gastrointestinal immune system is represented by the microfold (M) cells. M cells exhibiting microfolds on their luminal surface are responsible for absorption, transport, processing, and presentation of

antigens to subepithelial lymphoid cells. Major accumulations of lymphoid tissue are found in the lamina propria of the intestine. M cells in the intestinal epithelium overlying Peyer patches allow transport of antigens to the lymphoid tissue beneath it. The complex interplay among antigens, cells, and cytokines results in very efficient immune responses.

Subepithelial cells include $CD4^+$ type 1 T-helper cells (THCs) and IgD/IgM^+ B lymphocytes, the latter being antigen-presenting cells (APCs) and function as memory cells interacting with type 1 THCs. Together, this group of cells constitutes a "pocket" of M cells. Antigen-receiving DCs and macrophages interact with T cells in the GALT, thereby promoting indirectly the increase in mucosal IgA produced by activated B-cells. Bacterial invasion and adherence can be quenched via trafficking of IgA through epithelial cells into the lumen. Stimulation of B lymphocytes leads to the production of IgA and IgM within the Peyer patches [Beagley and Elson 1992, Dubois et al 1999, Greer et al 1999]. In addition, migration and maturation of cytotoxic T cells in the lamina propria serves as another vital tool for suppression of microbial assault [Nagler 2001].

1.4. Oxidative stress

Redox reactions and formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important signalling role in cell metabolism under normal physiological conditions. The reactivity of oxygen-containing free radicals such as hydroxyl radical, superoxide anion, lipidperoxyl radical and nitric oxide can be attributed to the presence of one or more unpaired valence shell electrons. The main source of ROS generation is the mitochondrial oxidative phosphorylation. In mitochondrium superoxide anions are formed as a result of respiratory electron transport chain operation giving rise to superoxide anions, which are quickly metabolized to hydrogen peroxide [Murphy 2009].

Unsaturated fatty acids, especially polyunsaturated fatty acids (PUFA), are very sensitive to ROS-mediated injury, that is why oxidative stress can lead to lipid peroxidation in biological membranes. Unstable radicals are formed as free radicals derived from unsaturated fatty acids (e.g. arachidonic acid, docosahexaenoic acid) of membrane phospholipids. As initiation of the lipid peroxidation cascade, generation of conjugated dienes (CDs) can be detected, while MDA will be produced only in terminal steps. After isomerization and molecular rearrangement via their double bonds these instable radicals through the transient stage of a conjugated dien structure become lipid hydroperoxides (**Fig. 3**). Meta-stable end-

product of lipid peroxidation, malondialdehyde (MDA) can be used as marker for tracing lipid peroxidation [Placer et al 1966, Matkovics et al 1988].
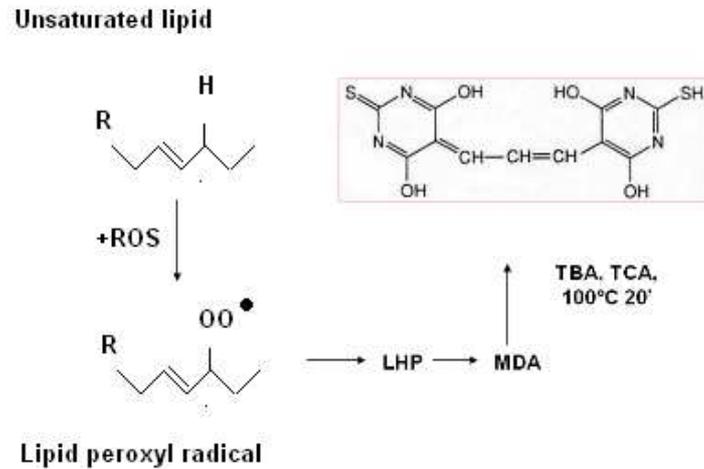
Fig. 3 Outline mechanism of lipid peroxidation under oxidative stress. Investigation of lipid peroxidation can be performed via thiobarbituric-acid (TBA)-based analysis through MDA formation using trichloroacetic acid (TCA) for protein precipitation.

The consequence of lipid peroxidation is the irreversible damaging effect on membrane structure leading to significant loss in barrier integrity and leakage of substances due to increased permeability [Valko et al 2007, Marnett et al 1999].
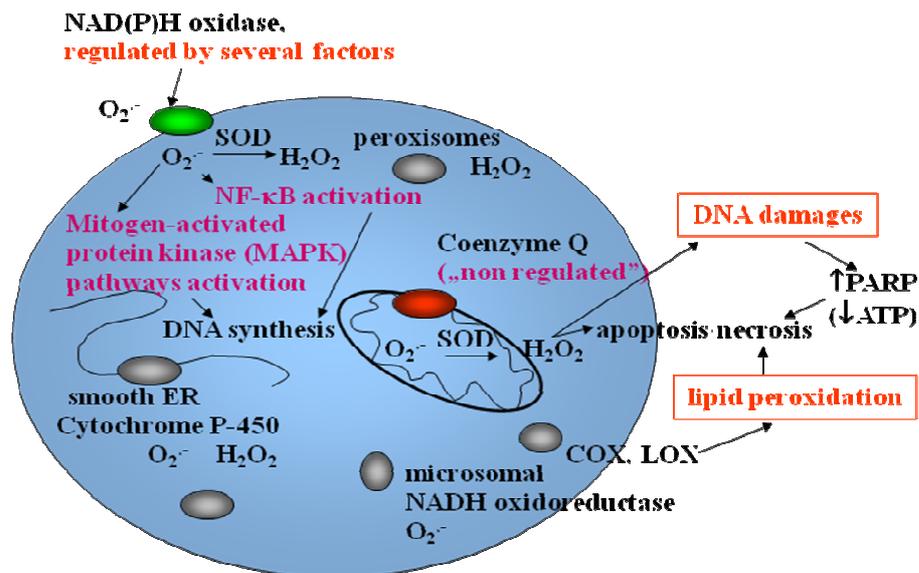
Fig. 4 Cellular sources and damaging effects of of reactive oxygen species.

ROS are formed intracellularly under physiological conditions, however in excessive amounts ROS propagate pathological changes such as protein oxidation and DNA damage (**Fig. 4**). The characteristic reaction of ROS is oxidation of sulfhydryl groups in cysteine residues resulting in protein dimerisation by intermolecular disulphide linkage [Finkel and Holbrok 2000]. Next to other ROS producing enzymes (xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450, NADPH-oxidase (NOX) is considered as major contributor to superoxide anion generation. Reduction of ROS is part of physiologically operating scavenging mechanisms maintained by an antioxidant defence system. Superoxide anion radicals are scavenged by superoxide dismutase (SOD) converting the substrate to hydrogen peroxide, which then further transformed into water and oxygen by catalase [Chance et al 1979]. The another antioxidant enzyme is glutathione peroxidase, which can govern reduction by the consumption of reduced GSH constantly recovered by glutathione reductase with the aid of H-donor, NADPH [Mannervick 1987].

The activity of this NOX was discovered in phagocytes in killing pathogenic microorganisms by the aid of superoxide anions in extracellular space. In non-phagocyte type cells several isoforms of NOX were observed and it was confirmed that superoxide anion in different cellular compartments [Hancock et al 2001] could play important role in cell signalling. Furthermore, it has been established, that different cell types react to extrinsic and intrinsic stimuli such as certain growth factors, cytokines and environmental factors generating low levels of ROS. ROS which at such a low dose act as second messenger related to cell proliferation, apoptosis and redox-sensitive signal transduction pathways of chronic inflammatory and associated degenerative processes. Prolonged oxidative stress and/ or excessive amounts of ROS tip the delicately controlled redox balance and it can induce programmed cell death, apoptosis, or in extreme cases in concentration-dependent manner necrosis. ROS can modulate protein tyrosine phosphatases such as mitogen activated tyrosine kinase (MAPK) thereby regulating transcription factors (c-myc, p38) responsible for apoptosis or cell differentiation. The inflammation–triggering effect of ROS can be attributed to activation of certain transcription factors (AP-1, NFκβ, HIF-1) [Waldeck et al 2009].

Two types of cell death are known: Necrosis is usually a passive process caused usually by pathological conditions accompanied by rapid and irregular desintegration of the cell and consequently uncontrolled leakage of the cell components causing inflammation [Majno and Jorris 1995]. In contrast, apoptosis takes place without cell swelling and

desinteration, and the apoptotic bodies surrounded by membrane are ingested by phagocytes. [Hu et al 1999].

The differentiated and undifferentiated cells may respond to the same dose and treatment time of peroxide with differential sensitivity to ROS. Different cell lines such as SH-SY5Y neuroblastoma cells, HL-60 human promyelocytic leukemia cells and mouse 3T3-L1 cells exhibit markedly different resistance to cellular stressors suggesting that profound differences may exist in mitochondrial metabolism or ROS-scavenging enzyme-driven antioxidant defenses [Covacci et al 2001, Kojima et al 2010, Schneider et al 2011].

1.5. Transepithelial electric resistance of intestinal epithelial cells

Currently, most *in vitro* intestinal models are cultured as one-dimensional monolayers on plastic surfaces. However, culturing epithelial cells on polycarbonate or polyester membrane inserts leads to spontaneous cell differentiation and polarization enabling basolateral feeding of epithelia similar to the *in vivo* setting through the polarised epithelial monolayer. The advantage of 3D polymer membrane insert-based pig intestinal cell model is its similarity with *in vivo* conditions and its applicability and accessability for bioavailability and transport studies [Cencic et al 2010]. Single cell cultures are good models to study phenomena of the epithelial cell type such as bacterial cell adhesion, ion transport or response to extrenal stimuli exerted by ROS.

Porcine intesinal epithelial cell line, IPEC-J2 forms a single cell monolayer consisting of cuboidal cells interspersed with flat cells without goblet cells. It was confirmed based on electron microscopic images that apical microvilli are grown with different lengths and widths and due to the function of apical junctional complexes IPEC-J2 cells become polarized. Immunostaining revealed TJ proteins such as claudin-3 and claudin-4 co-localized with occludin in the apicolateral membrane of all cells. The integrity of monolayer can be followed measuring transepithelial electrical resistance between apical and basolateral compartment of the IPEC-J2 cells. Cell membranes and superimposed thin, extracellular glycocalix layer (mucopolysaccharide) can also be observed [Schierack et al 2006]. In IPEC-J2 cells expression of mRNAs encoding the cytokines IL-1α, IL-6, IL-7, IL-8, IL-18, TNF-α and GM-CSF, but not TGF-β or MCP-1 was detected.

The major advantage of IPEC-J2 cells compared to the most widely used colon carcinoma cell lines Caco-2 and HT-29, is that their glycosylation pattern, proliferation rate

and colonisation ability characterize better the *in vivo* conditions in the gut ecosystem. The high TER value of IPEC-J2 monolayers grown on Transwell collagen-coated PTFE filters (~2000 Ohm*cm^2) and on Transwell polyester filters coated with rat tail collagen (~6000 Ohm*cm^2) demonstrates the functional integrity of the continuous cell association, acting as a single-layer tight physical barrier. Decrease in TER may reflect an increase in movement of solutes and ions across the intestinal epithelium. It was reported that TER of filter-grown Caco-2 cell mono-layers was reduced by 5%, 10%, 15% and 33% in concentration-dependent manner 1 h after application of 0.5 mM, 1 mM, 5 mM and 10 mM H_2O_2. The absence of H_2O_2-induced lactate dehydrogenase (LDH) release indicated that the decrease in TER by the addition of H_2O_2 to the apical compartment was not the cause of cell lysis [Rao et 1997]. Consistent with these findings, hydrogen peroxide also increased the permeability with a similar pattern in airway epithelial cells [Chapman et al 2002] and in bovine brain microvascular endothelial cells [Lee at al 2004]. Furthermore, it was reported that in Madin-Darby canine kidney (MDCK) type II epithelial cell lines exposed to 5 mM H_2O_2 transepithelial electrical resistance (TER) was reduced to 23% of control, but TER returned to baseline within 6 h [Meyer et al 2001].

1.6. The role of junctional complexes in intestinal epithelium

Altered pattern of TJ and adherent junction proteins was described in normal tissues exposed to oxidative stress and also in inflammatory mechanisms and proliferative disorders. These proteins can act not only as static physical barrier, but they have also unique function in establishment of a dynamic interplay with the surroundings. The importance of TJ proteins lies in propagation of cell polarization and paracellular transport in addition to crosstalk with the microenvironment. The basis of enterocyte barrier function is epithelial cell-cell adhesion: The components of intercellular junctional complexes are TJs, adherens junctions (AJ, zonula adherens), gap junctions and desmosomes [**Fig. 5**]. Cell cytoskeleton-extracellular matrix connection can be constituted via transmembrane cell adhesion proteins, integrins. In addition to its basic function for enabling weak attachment of the cells to their surroundings these molecules can activate intracellular signalling pathways. Two main types of transmembrane proteins are found in TJ, occludin and claudins, which connect adjacent enterocytes. TJ also contains intercellular zonula occludens (ZO), which links the transmembrane junctional proteins to the actomyosin cytoskeleton and cytoplasmic regulatory proteins in addition to its role of binding TJ to AJ [Ohland et al 2010].

In epithelial TJs, claudins are considered as key integral protein regulators responsible for maintenance of electrical resistance, paracellular ionic selectivity and transport mechanisms in epithelial and endothelial structures. There are currently at least 24 known members of the claudin family, which are expressed in a tissue specific pattern [Gonzalez-Marriscal 2003, Oliveira and Morgado-Diaz 2007]. Claudin-1 is present in tighter segments, high resistance epithelia such as distal and collecting duct of the nephron [Reyes et al 2002]. In MDCK epithelial cells, overexpression of claudin-1 was associated with a significantly higher TER [Inai et al 1999, McCarthy 2000]. There is also emerging evidence indicating that claudin-4 and claudin-7 are also involved in the barrier function of epithelial cells [Hou et al 2006]. Claudin-4 expression reduced paracellular electrical conductance through a selective decrease in sodium permeability without a significant effect on chloride permeability and flux for a non-charged solute [Van Itallie 2001]. Claudin-3 degradation associated with oxidative stress-induced changes in epithelial permeability was revealed using the human gastric carcinoma cell line MKN28. Furthermore, rebamipide, a radical scavenger, prevented epithelial barrier dysfunction by attenuating the H_2O_2-induced decrease in claudin-3 [Hashimoto et al 2008].
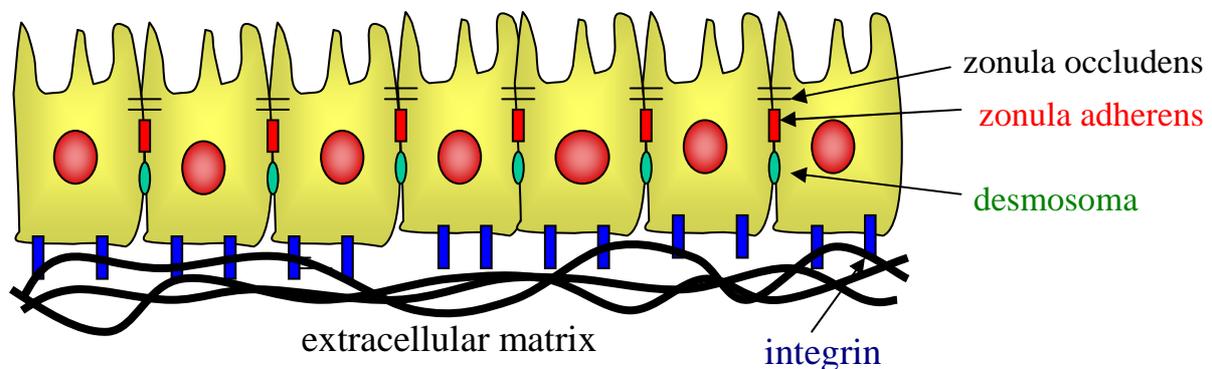
Fig. 5 Junctional complex assembly structuring enterocytes into cell monolayer

Transcellular permeation of gentamicin is practically not possible in view of its relatively high molecular weight, and highly polar and hydrophilic nature [Rama Prasad et al 2003). Paracellular permeation across TJs, however, might occur [Madara et al 1989] and peroxide can facilitate this process by modulating protein assembly in TJs. Pro-inflammatory factors such as enteroinvasive *Escherichia coli*, oxidative stress induced by xanthine oxidase and xanthine or H_2O_2 and pro-inflammatory cytokine TNF-α have been shown to cause rearrangement and decreased expression of TJ and AJ proteins in Caco-2 and MKN28 cells

[Seth et al 2008, Miyauchi et al 2009, Qin et al 2009]. This was also found in dextran sodium sulfate-induced colitis in mice [Menigen 2009]. Structurally, gentamicin is a 4,6-disubstituted aminocyclitol composed of the core aminocyclitol moiety, 2-deoxystreptamine (2-DOS), to which the amino sugars, purpurosamine and garosamine are bound at positions C-4 and C-6, respectively (**Fig. 6**).
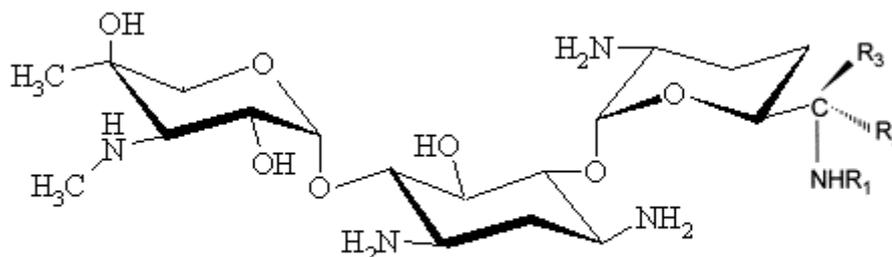
Fig. 6 Chemical structure of gentamicin complex: C_1, C_{1a}, C_2 and C_{2a}. The side chains are the followings: gentamicin-C_1: R_1 -CH_3, R_2 –H, R_3-CH_3, gentamicin-C_{1A}: R_1, R_2, R_3 –H, gentamicin-C_2: R_1, R_2 -H, R_3-CH_3, gentamicin-C_{2A}: R_1, R_3: -H, R_2 –CH_3

In its therapeutic form, gentamicin comprises a complex of gentamicin C_1, C_{1a}, and C_2, which differ only in the degree of methylation of the C-6′ position of the sugar attached at C-4 of 2-DOS [Testa and Tilley 1976]. Other component of gentamicin is C_{2a}, which is a 6'-C epimer of C_2 [Seidl and Nerad 1988]. Several papers on high-performance liquid chromatographic analysis [Isoherranen et al 2000, Soltes 1999, Lacy et al 1988, Stead and Richards B 1996, Posyniak et al 2001, Al-Amoud et al 2002] with fluorometric detection have been published for quantitative determination of components of the gentamicin complex which are closely related compounds C_1, C_{1a}, C_2 and C_{2a}. For derivatization, o-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC) are frequently used to detect gentamicin in complex biological matrices.

1.7. Characterization of intestinal microbiota

Protection against pathogenic invasion, immunmodulation, nutrient absorption and processing, metabolic activity belong to versatile physiological functions of intestinal

microbiota. Homeostasis of gut ecosystem is affected by several extrinsic and intrinsic factors such as genetic susceptibility of the host, microbial population, host immune reactions and environmental factors. Humans as well as animals are in contact with a vast multitude of bacteria at epithelial surfaces of the body. The intestinal tract harbors 10^{13}-10^{14} microorganisms of more than 500 different species of bacteria. There is a progressive increase in the number of bacteria along the small intestine, from approximately 10^2-10^4 in the jejunum mainly *streptococci* and *lactobacilli* to 10^7 colony-forming units (CFU) per gram of luminal content such as *streptococci, actinomycinae*, *corynebacteriae, clostridium* at the distal ileum. Anaerobes are predominant in the colon, and bacterial counts reach around 10^{12} CFU per gram of luminal content [Guarner 2005].

There are two types of bacterial populations in the gastrointestinal tract: Native bacteria are primarily acquired at birth and during the first year, and they are permanent gut residents (including commensal bacteria). On the other hand, transient microflora is supplied from external environment and diet. Bacterial colonization at early stage also depends on genetic traits. The sterile fetal gut gets inhabited by enterobacteria species such as *E. coli* and *Bifidobacterium* after birth [Kaser 2010]. The microbial colonization of the GI tract of a infant is influenced by milk-feeding and weaning. After weaning more stable microbiota starts to develop. Along the gastrointestinal tract pH value varies from stomach (pH=1.5-5), through the small intestine (duodenum pH=5-7, jejunum pH=7-9, ileum pH=7-8) to the colon (pH=5-7). Anatomically the large intestine consists of the cecum, ascending colon, transverse colon and descending colon, sigmoid colon and rectum. The main target site of bacterial metabolic activity and carbohydrate fermentation is the ascending colon, where the pH value is generally lower (pH=5-6) compared to that of distal colon due to the carbohydrate fermentation and simultaneous production of SCFAs [Guarner and Malagelada 2003, Vigsnaes 2011].

1.8. Pre- and probiotics, synbiotics

Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts (World Health Organization/ Food and Agriculture Organization 2001). Probiotics are supposed to meet five main criteria: In addition to their beneficial effect on host ecosystem, they are without toxic effects and pathogenicity, they should be present in viable form in large quantity and they should preserve capability of survival, reproduction, intestinal metabolic activity and possess prolonged shelf-life. They are

recommended for the recolonization and are supposed to have positive influence by modification and support of physiological metabolism of the large intestine. Bifidobacteria and lactic acid bacteria (LAB) are the common microorganisms used as probiotics. However, some yeasts and bacilli can be found in some probiotic products (**Table 1**).

Probiotics have already proven their therapeutic values in the prevention and treatment of several intestinal infections, but the mechanisms by which they modulate the immune system is poorly understood. In addition, the therapeutic and prophylactic effects of probiotics on various diseases depend on the strains, administration routes, doses and the progression rate of the diseases. Host physiology, performance and farm productivity are largely influenced by changes in three components of gastrointestinal tract microbial ecosystem such as microbial community, crosstalk between host and microbiota and the nutrient source, the diet. Dietary inclusions of functional feed ingredients (probiotics, prebiotics or synbiotics) can be a valuable nutritional strategy in animal production, growth promotion and performance enhancement [Berg et al 1996].

Table 1 Summary of characteristic features of some probiotics

Probiotics	Genus	Fermentation type	Metabolites in broth	Reference
Lactobacillus plantarum 2142	Lactobacillus	heterofermentative	lactic acid, acetic acid, succinic acid butyric acid valeric acid	Zalan et al, Eur Food Res Technol 2010
Lactobacillis casei Shirota	Lactobacillus	homofermentative	lactic acid butyric acid	Zalan et al, Eur Food Res Technol 2010
Bifidobacterium animalis subsp. *lactis BB-12*	Bifidobacterium	heterofermentative	acetic acid, lactic acid, formic acid	van der Meulen et al, Appl Environ Microbiol 2004
Bacillus amyloliquefaciens	Bacillus		BLIS, amylolysin, subtilisin	Abriouel et al, FEMS Microbiol Rev 2011
Enterococcus faecium	Enterococcus	homofermentative	lactic acid	Bulut et al, J Dairy Res 2005

BLIS: bacteriocin-like inhibitory substances

Prebiotics are non-digestible food/feed constituents, which can promote benefit to host through selectively promoting the growth or/and activity of commensal bacteria. They are supposed to improve health via the nutritional manipulation of the intestinal microbiota ecosystem. They are defined as selectively fermented ingredients which cause specific changes in composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health [Robertfroid 2007]. By avoiding digestion due to their resistance to degradation by gastric acid and the digestive enzymes they are reaching the proximal and to some extent the distal colon without decomposition, where they can be selectively metabolized by colonic bacteria [Fooks and Gibson 2002, Langlands 2004, Rastall 2002]. The majority of prebiotic candidates are poly- and oligosaccharides, which are either extracted from plant tissue or synthetized enzymatically. Plant-derived polysaccharides such as inulins possess different molecular weight profiles depending on degree of polymerization from 3 to 70 [Sirisansaneeyakul 2007]. Prebiotic non-digestible oligosaccharides usually contain 3-10 sugar moieties. All recognized prebiotics contain saccharide units, but only carbohydrates with FOS, inulin, lactulose and galacto-oligosaccharides (GOS) are marketed in EU as food or feed additives.

Inulin and FOS can not be digested by the enzymes of the mammalian small intestine. It was found that inulin can stimulate the generation of butyrate and it can propagate the growth of lactid acid bacteria in the colon of healthy individuals [Videla 2001, Schneeman 1999]. The efficacy of germinated barley foodstuff (GBF) dietary fiber fraction in the treatment of rat DSS colitis was proven by monitoring decrease in serum IL-8 level and stimulation of butyrate dependent anti-inflammatory mechanisms by induction of luminal butyrate-production [Andoh 1999]. As a potential prebiotic, xylo-oligosaccharides (XOS) were capable of increasing the amount of bifidobacteria throughout all segments of the intestine, especially in ileum, as was shown in a mouse model. Via modulation of SCFA production and attachment of G protein-coupled receptor 43, (GPR43) [Maslowski 2009] to its substrate on neutrophils, XOS can downregulate the level of pro-inflammatory cytokine IL-1β. The beneficial effect of XOS on reduction of systemic and mucosal inflammation appears to be due to elevated number of intestinal bifidobacteria and increased SCFA production [Metzdorff 2012].

Synbiotic is a mixture of pre- and probiotics. Anti-inflammatory and immunmodulatory effect of synbiotics, especially multistrain/multifiber type is manifested in facilitation of secretory IgA production and elevated IL-10 in cecum. A pioneering research in which the differences in proteomes of *Bifidobacterium animalis* subsp. *lactis* BB-12 are

compared when probiotics are cultured and fed on glucose or XOS containing matrices. It was revealed that proteins secreted from *Bifidobacterium animalis* subsp. *lactis* BB-12 grown on XOS may have role in the colonization of the GI tract, adhesion to host tissues or modulation of the host immune system [Gilad et al 2010, Gilad et al 2011].

The symbiotic microbes have profound influence on physiology, immunology and nutrition of animals and humans. Loss of dynamic equilibrium between microbes and host could be a serious risk factor in inflammatory bowel disorder, metabolic syndrome, autoimmune diseases and colonic carcinogenesis. Maintenance of gut microbial homeostasis via symbiotic microbes involves a direct protective effect on host or modulation on intestinal microbiota in a beneficial way. The gastrointestinal tract gets colonized after birth by symbiotic microbes from the mother and from the environment such as nutrient sources, until a stable, diverse and complex ecosystem is formed. The microbial community and its activities exert remarkable effects on the health and performance of farm animals.

Proposed mode of action of probiotic microorganisms (**Fig. 7**) against pathogenics in gut lumen is dependent on close interaction by aggregation (auto-aggregation and co-aggregation), adhesion to epithelial cells and extracellular matrix and physical barrier effect of probiotic strains in the form of pathogenic exclusion. [Quigley 2010, Zareie 2006, Mattar et al 2001]. To avoid the penetration of pathogenics through the intestinal wall the probiotics are capable of reinforcement of TJ assembly between enterocytes.
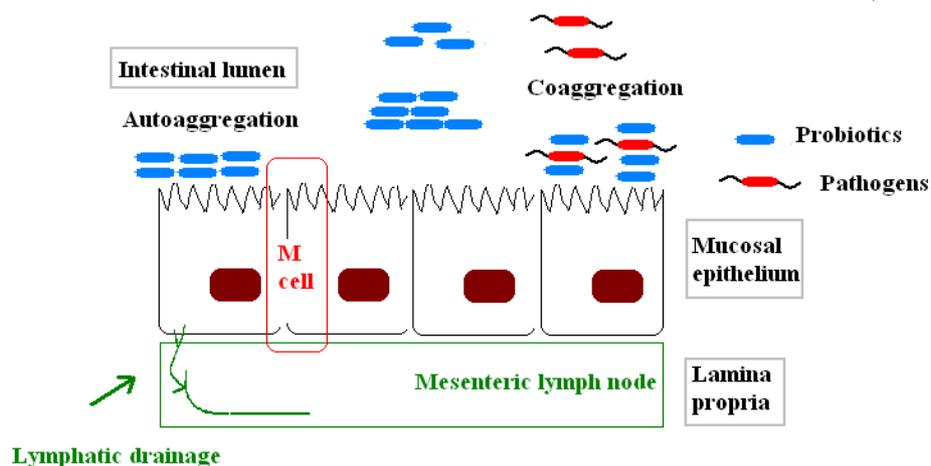
Fig. 7 Multifaceted mode of probiotic actions: Probiotics can coaggregate with pathogens to inhibit their adhesion. Biofilm formation via autoaggregation lowers the risk of pathogen colonization on epithelial surface. Probiotics can also reinforce mucosal epithelium barrier against enterotoxic pathogen invasion.

Some bacterial strains stimulate the synthesis of zonula occludens-1 (ZO-1) and occludin in enterocytes via TLR-2 signalling [Karczewski 2010], other probiotics inhibit the the opening of TJs by blockage of apical cytoskeleton constriction [Ait Belgnaoui et al 2006]. Therapeutic importance of probiotics in human and veterinary medicine lies in the treatment of pathogen-induced inflammation and in elimination of side effects caused by prolonged per os administration of antibiotics. The physiological equilibrium in gut microbiota can be maintained by probiotics if they are taken up in appropriate amount with their ability to occupy available sites [Kyriakis 1999].

Intestinal immune system is also provoked by probiotics via components of GALT such as Peyer patches, lymphocytes of lamina propria by inducing IgA secretion [Shanahan 2002, Reid et al 2003, Isolauri et al 2001]. Biologically active molecules such as bacteriocins possessing antimicrobial properties are produced by probiotics [Corr et al 2007]. Other products affect inflammatory responses in surrounding cells: they inhibit the overproduction of proinflammatory cytokines while the anti-inflammatory cytokine level remains the same [Gareau et al 2010, McCarthy et al 2003].

1.9. Gut ecosystem: The importance of probiotics in animal health

As the use of low-dose dietary antibiotics for growth promotion in livestock has been banned in EU due to the widespread occurence of resistance against pathogens. Nowadays it is a growing interest to replace antibiotics by probiotics. The common feature of these live microbial food/feed ingredients, the probiotics is that they exert beneficial effects on host health and well-being. Probiotics have already proven their therapeutic values in the prevention and treatment of several intestinal infections, but the mechanisms by which they modulate the immune system is poorly understood. In addition, the therapeutic and prophylactic effects of probiotics on various diseases depend on the strains, administration routes, doses and the stage of the disease. Host physiology, performance and farm productivity are largely influenced by changes in three components of gastrointestinal tract microbial ecosystem, namely (i) microbial community (ii) crosstalk between host and microbiota and (iii) the nutrient source, the diet. Dietary inclusions of functional feed ingredients (probiotics, prebiotics or synbiotics) can be a valuable nutritional strategy in animal production, growth promotion and performance enhancement [Berg et al 1996].

1.10. Role of cytokines and heat shock proteins in inflammation

Cytokines are small regulatory proteins mainly secreted by immune cells playing active part in intercellular communication. Due to their immunmodulatory mode of action they influence the activity of the native and the acquired immun system and they coordinate the inflammatory response [Vilcek 2004]. Cytokines include interleukins, interferons, tumor necrosis factor- α (TNF-α) and β and non-immunological cytokines such as erythropoietin. A general characteristics of cytokines is redundancy (different cytokines with similar function) and pleiotropy (certain probiotics with different properties) [Ozaki et al 2002]. Cytokines bind to specific membrane-based receptors and they induce an intracellular signalling cascade impacting the gene expression profile. They have regulatory effect on their own receptor distribution (upregulation or through negative feedback downregulation). The role of cytokines in inflammatory processes has been intensively studied area. Certain cytokines have been categorized as proinflammatory ones due to their inflammation-inducing properties such as IL-1, IL-6, IL-8 and TNF-α in contrast to the anti-inflammatory cytokines, IL-4, IL-10, IL-11, IL-13 [Dinarello 2000]. However, in accordance with the pleoiotrop nature of cytokines IL-4, IL-10 and IL-13 can activate B-lymphocytes and at the same time they can suppress the genes responsible for the production of IL-1, TNF-α in the cells. Oxidative stress can lead to the formation of inflammatory cytokines [David et al 2007]. Intestinal epithelial cells exposed to acute oxidative stress can secrete IL-1β, IL-6, IL-8 and TNFα [Son et al 2005].

Heat shock proteins (Hsp) are responsible for protecting cell proteins from malfunction and denaturation evoked by harmful stimuli such as heat treatment and chemical intervention. Hsp gene expression can dramatically increase within a short period of time if for instance the cells are exposed to elevated temperature or oxidative stress [Santoro et al 2000, Borges et al 2005, Musch et al 1996]. Hsps are supposed to downregulate gene expression of inflammatory cytokines and to block NF-κβ or MAPK-triggered inflammation, but the exact modes of action remain to be elucidated [Petrof et al 2004]. There is some evidence that administration of *Lactobacillus* spp. supernatant can induce the synthesis of Hsp 70 in both crypt- and villus-like Caco-2 cells suggesting one putative mechanism behind the beneficial properties of non-starter lactobacilli on host defence against infection and inflammation [Malago et al 2010, Nemeth et al 2006] (**Fig. 8**).
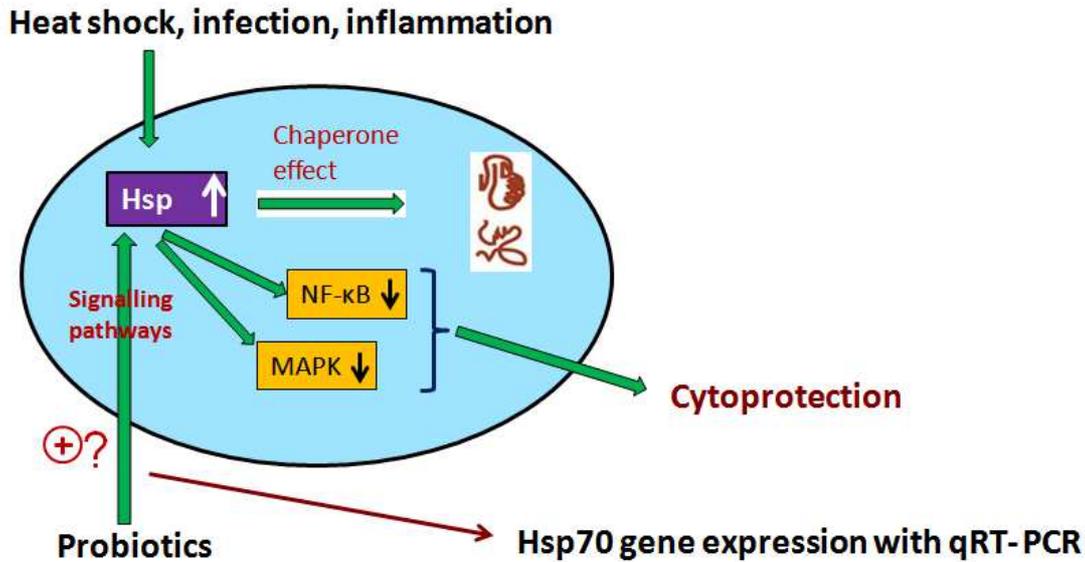
Fig. 8 Probiotics may confer anti-inflammatory and cytoprotective action via inhibition of external stimuli (heat shock, infection and oxidative stress)-induced NF-κβ alteration and increase of Hsp production as novel mechanisms of microbial-epithelial interaction.

The most frequently used probiotics are Gram-positive, facultative or obligate anaerobic bacterial species, which belong to the *Lactobacillus* and *Bifidobacterium* genus. More probiotics can be found among *Bacillus, Streptococcus, Enterococcus, Pediococcus*, *Lactococcus* genus and among yeasts [Gaggia et al 2010]. Application of most probiotic strains is considered to be safe. However, certain strains of *Enterococcus* seem to be capable of transmitting antibiotical resistance to other bacteria. In addition, probiotic candidate, *Bacillus* species should also be administered under caution due to the production of enterotoxins [Anadon et al 2006]. Nonstarter, facultative, heterofermentative lactobacillus strain, *Lactobacillus plantarum* 2142 secretes lactic acid, tartaric acid, acetic acid in higher amount [Zalan et al 2010], and it can inhibit the growth of several bacterial strains such as *Bacillus cereus*, *Listeria monocytogenes* and *Salmonella enteritidis* 857.

1.11. Fermentation and metabolic byproducts

Bacterial fermentation products include SCFAs primarily acetate, propionate and butyrate in addition of the presence of intermediate compounds such as lactate, pyruvate, ethanol and succinate. SCFAs are produced by bacterial cells from monosaccharides degraded by hydrolysis from poly- and oligosaccharides. Production of pyruvate and acetyl-CoA is

important milestone in the formation of fermenative metabolic molecules such as acetate or butyrate. Glucose can be fermented by *lactobacilli* through two major pathways via glycolysis (Embden-Meyerhof pathway) in homofermentative manner or via 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway used by heterofermentative LAB. Lactic acid is the main metabolites of both reactions, but the stoichiometric ratio of lactate produced per glucose is 2 in glycolysis in contrast to 6-PG/PK pathway, where only 1 mole lactate is produced from 1 mole glucose. The appearance of acetate and lower ATP yield can also be observed in the latter pathway [Axelsson 1998, Zalan et al 2010].

The spectrum of produced metabolic byproducts depends on the consumed carbohydrate type. Acetate is the main metabolite of arabinan and pectin hydrolysis [Al-Tamimi et al 2006]. Acetate and propionate are produced in excess, when arabinogalactan is fermented [Englyst et al 1987] and butyrate is formed in high amount as a result of fructan digestion [Karpinnen et al 2000]. SCFAs such as acetate, proprionate and butyrate are absorbed and metabolized by IECs, liver and muscles exert their effect on inhibition of TNF-α-mediated activation of NF-κβ signalling in human adenocarcinoma cell lines and induce anti-inflammatory activity in an *in vitro* model of murine experimental colitis [Tedelind et al 2007]. Histone deacetylase (HDAC) inhibitor n-butyrate is one of the SCFAs produced in the large intestine as a consequence of anaerobic microbial degradation of dietary fibers, undigested starch and proteins [Cummings 1981, Bugaut and Bentejac 1993, McIntyre 1993, Mcintosh 1996, Whiteley 1996]. Na-n-butyrate containing feed additives in different species was reported to regulate restoration of healthy intestinal microbiota [Galfi and Bokori 1990, Manzanilla et al 2006, Fernandez-Rubio 2008]. The acetylation and deacetylation of chromosomal histone proteins plays an important role in regulation of gene expression, in cell proliferation, induction of cell death [Gray and Ekstrom 2001]. Modulation of gene expression involving the regulation of proinflammatory cytokines by sodium butyrate seems to be a mechanism of its putative anti-inflammatory effect [Saemann 2000].

1.12. Pattern-recognition receptors: Toll-like receptors

Toll-like receptors expressed in epithelial and phagocytic cells contain a family of pattern-recognition receptors, which can detect highly conserved structures of pathogenicic and commensal bacteria and their molecular products such as LPS and lipoteichoic acid (LTA), which are recognized by TLR2 and TLR4 [Andoh 2006]. There is a definite role of NF-κβ pathway in the mucosal immune system in different cellular compartments. Increased

activation of NF-κβ with elevated expression of proinflammatory cytokines such as IL-6 and TNF-α in macrophages and IECs indicates inflamed mucosa and accelerated inflammatory events [Vallabhapurapu et al 2009, Neurath 1996]. MyD88 is an adaptor protein for TLRs, it participates in recruitment of IRAK-4, which becomes activated by phosphorylation via IRAK-1, which then increases activity of NF-κβ and MAP kinases [McGettrick 2004, Gohda 2004]. However, it is not fully understood how the TLRs of host microorganisms can make distinction between ligands derived from pathogens and commensal bacteria. Intestinal function is fine tuned by bacterial recognition, and therefore a disturbed microbiota homeostasis could contrubute to promoting chronic inflammation and cancer [Abreu 2010].

Using two colon adenocarcinoma cell lines, Caco-2 and T84, it was found that H_2O_2 could increase intestinal epithelial permeability leading to disruption of paracellular junctional complexes presumably via a protein tyrosine phosphorylation (PTP)-dependent mechanisms [Rao et al 1997]. Protein kinase C (PKC) isoforms in the gastrointestinal epithelium are considered as pivotal modulators of membrane dynamics, transepithelial permeability, epithelial responses to inflammatory mediators, ion secretion and barrier integrity through intracellular pathways. PKC inhibitors could markedly block TJ reassembly which raises the possibility that phosphorylation of TJ proteins may be important for their incorporation into the TJ during recovery from oxidative stress [Meyer et al 2001]. Three main groups of PKC isoenzymes exist: the classical type PKCs, PKCα, PKCβI, PKCβII, PKCγ (cPKC), non-classical PKCs PKCδ, PKCε, PKCθ, PKCη (nPKCs) and the atypical PKCs PKCλ, PKCζ and PKCι. The maintenance of barrier function of intestinal epithelial cells seems to be associated with homeostatic coordination via diacylglycerol-dependent (DAG) classical and calcium- and DAG-independent "atypical" protein kinases [Farhadi et al 2006]. There is an emerging evidence that TLR-2, TLR-4 and TLR-9 may regulate intestinal epithelial barrier integrity possibly via PKC isoenzymes-mediated downstream signalling pathways as major part of *lactobacilli*–conferred anti-inflammatory action [Karczewski et al 2010, Cario et al 2007, van Baarlen 2009, Grabig et al 2006, Rachmilewitz et al 2004].

2. Materials and methods

2.1. Cell lines and culture conditions

IPEC-J2 a non-transformed porcine intestinal epithelial cell line was a kind gift from Dr. Jody Gookin, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA. The cells were grown and maintained in complete medium containing 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixure (DMEM/F12) supplemented with 5% foetal bovine serum, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor and 1% penicillin-streptomycin (all from Fisher Scientific, USA). Cell cultures were tested by PCR according to van Kuppeveld et al. (1996) and were found to be free of mycoplasma contamination. For the experiments, IPEC-J2 cells between passages 42-48 were seeded onto six-well Transwell polycarbonate and Transwell polyester membrane inserts (Corning Inc., Corning, NY, USA), the latter coated with 8 μg/cm^2 rat tail collagen type I (Sigma-Aldrich, Steinheim, Germany) at a density of 1.5×10^5 cells/ml (1.5 ml apical volume and 2.6 ml basolateral volume per well was applied according to the manufacturer's instructions (**Fig. 9**).

Two human colon adenocarcinoma Caco-2 cell lines of different origins were investigated: Caco-2H was obtained from Dr H. Hendriks (Faculty of Veterinary Medicine, Department of Pathology, Utrecht University, The Netherlands) and Caco-2P was provided by Dr G. Mózsik (Department of Internal Medicine, Medical University of Pécs, Hungary). Caco-2 cells were grown in DMEM supplemented with 1% (v/v) MEM non-essential amino acids, 10 mM NaHCO$_3$, 4 mM glutamine, 50 μg/ml gentamicin sulphate, 25 mM HEPES, 1mM sodium pyruvate, 10% (v/v) heat inactivated fetal bovine serum (Gibco). Filter grown Caco-2 cells were cultured for 21 days to achieve a fully differentiated, completely polarized cell population. After 3 weeks, 1.5×10^6 cells were present on filter inserts.

Cells were allowed to adhere for 24 h before being washed and re-fed every other day until confluence. The cells were grown at 37°C in a humidified atmosphere of 5% CO$_2$. TER measurement of mono-layers was performed on alternate days after seeding from day 5 to 21 of culture using EVOM Epithelial Tissue Volt/Ohmmeter (World Precision Instruments, Berlin, Germany). IPEC-J2 cells were used for further experiments if the TER of cell monolayers reached 8000 Ohm*cm^2, which occurred routinely after 17 days. Maximal resistance of Caco-2H cells was 1000 Ohm*cm^2 and Caco-2P monolayers were allowed to reach TER values of ≥ 6000 Ohm*cm^2 prior to experiments.
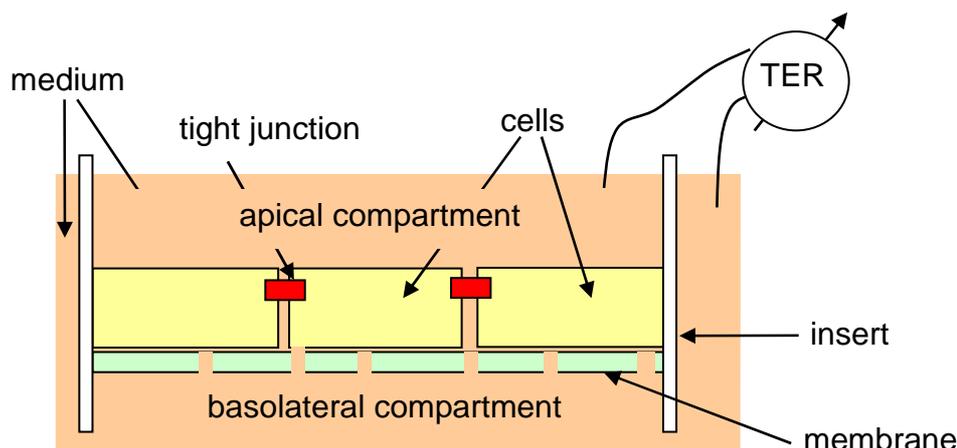
Fig. 9 Polyester filters and cell culture monolayer fully separating the apical and basolateral compartments. Diffusion between these two compartments is only possible via transcellular or paracellular transport.

2.2. Exposure of epithelial cells to H_2O_2

Before treatments confluent mono-layers of IPEC-J2 cells were washed twice with complete medium without antibiotics and foetal bovine serum (plain medium). The H_2O_2 solution was prepared freshly prior to each experiment. Different concentrations of H_2O_2 (0-4 mM)/ plain medium were administered apically for 1 h in order to study the dose-response of the cells. Time-course response was also investigated when IPEC-J2 cells were treated with peroxide for different time intervals (0, 15, 30, 45 and 60 min). Time course of cytokine expression induced by a 1 h treatment with 1 mM peroxide was determined during a 24 h period. After the incubation period with peroxide, cells were washed twice with plain medium before being subjected to the subsequent procedures. TER measurements were performed both before and after the H_2O_2 treatment.

2.3. Determination of H_2O_2 decomposition and assay of hydrogen peroxide in the presence of bacterial SCSs

Concentration of H_2O_2 in aqueous solutions was determined by a colorimetric method according to Nowak (1990). Briefly, for determination of peroxide decomposition rate, ten ml aliquots of bidistilled water or plain medium without phenol red containing 0.5 mM-1 mM H_2O_2 (sample) or without peroxide content (control) were prepared. Five-hundred μl of samples were taken after 0, 15, 30, 45 and 60 min of incubation at 37°C; diluted five-fold, and

mixed with 0.05 ml of 1% aqueous o-dianisidine (Sigma) and 0.5 ml of 0.001% aqueous horseradish peroxidasese (Sigma). Concentration of H_2O_2 in aqueous solutions was quantified by the same colorimetric method in the absence and in the presence of SCS of probiotics when the antioxidant capacity of SCSs was evaluated. The blank contained plain phenol red-free DMEM with or without bacterial growth media.

The samples were incubated for 10 min at 37°C. The reaction was stopped by adding 0.1 ml of 4 M HCl to each tube. H_2O_2 concentration of each sample was determined at 400 nm wavelength by the Cary 50 UV-Vis spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Australia) using a 5-point standard calibration curve.

2.4. Determination of H_2O_2 uptake

IPEC-J2 cells were exposed to 1 mM H_2O_2 suspended in plain medium without phenol red for 0, 15, 30, 45 and 60 min. Control cells were treated with plain medium without phenol red. One ml sample was taken from the apical and basolateral compartments for H_2O_2 assay and measured as described above.

2.5. DAPI staining

To elucidate the effect of H_2O_2 treatment on the viability of IPEC-J2 cells, they were exposed to different concentration of H_2O_2 (0, 0.5, 1, 2 and 4 mM). After treatment for 1 h, H_2O_2 was removed, cells were washed with 1 ml plain medium twice and stained with 1 μg/ml DAPI (4',6-diamidino-2 phenylindole, Sigma)/PBS for 10 min. Cells were then washed five times with 2 ml PBS and fixed with 4 v/v% formaldehyde/PBS solution. Until the microscopic analysis, the samples were covered with glycerol/PBS (3/1) and stored at 5°C. In order to determine the proportion of dead cells at least ten microscopic fields were evaluated at each concentration.

2.6. Cytotoxicity evaluation with TB and NRU

Neutral red uptake assay is suitable method for investigation of cell viability in the absence and in the presence of Ca^{2+} ions (1.05 mM). This method is based on the ability of living cells to incorporate and bind the supravital dye, neutral red in the lysosomes [Repetto et

al 2008]. Undifferentiated IPEC-J2 cells were seeded in 96-well tissue culture plates and they were treated with either 0.5 mM H_2O_2 or sodium butyrate in the presence of peroxide for an h. After thorough and repeated washing with phosphate buffered saline (PBS), the plates were incubated for 2 h with a medium containing neutral red at 50 µg/ml. Cells were subsequently washed with PBS. and then the dye was extracted in each well with neutral red destain solution containing 50% ethanol (96%), deionized water (49%) and 1% glacial acetic acid. Absorbance was measured at 540 nm in a microtiter plate reader spectrophotometer (Dynex Relevation 4.22) using blanks which did not contain cells.

Trypan blue staining was applied to percentage of dead cells based on their ability to take up trypan blue dye. Living cells with intact cell membranes exclude uptake of the diazo dye. Undifferentiated IPEC-J2 cells were treated with 0.5 mM hydrogen peroxide and sodium butyrate in the presence of peroxide for an 1 h. After washing the cells with PBS twice, the staining solution containing 0.4% TB and PBS (1:7) was added. Incubation of the controls and the samples with the dye was for 15 min at 37°C. After removal of the staining solution and rinsing with PBS, the ratio of the dead cells *versus* living cells was determined by cell counting.

2.7. Immunohistochemistry

Five-five samples have been removed from the jejunum of the carcasses of unsuckled 1-day-old newborn and adult swine (n = 10). Following excision the tissue samples were fixed in 8% neutral buffered (in PBS, pH 7.0) formalin solution for 24 hs at room temperature, dehydrated in a series of ethanol and xylene and embedded in paraffin. The 3-4 µm thick sections were routinely stained with hematoxylin and eosin. Our work has been in accordance with veterinary law and ethical regulations. IPEC-J2 cells on mebrane inserts were subjected to the same preparation procedure except that they were fixed in 4% neutral buffered formaldehyde solution. The hematoxylin-eosin stained slides were examined and only the intact, not autolysed and inflammation-free samples were chosen for further investigations. For immunohistochemistry 3-4 µm thick sections were cut and stained with antibodies to E-cadherin, claudin-1, -4, and -7. The sections were dewaxed in xylene and graded ethanol. After treatment with appropriate antigen retrieval solution (Target Retrieval Solution, DAKO, Glostrup, Denmark, pH 6; microwave oven for 30 min) the sections were incubated with antibodies against anti-claudin-1 (diluted 1:100, rabbit polyclonal, Zymed Laboratories Inc., San Francisco, CA, USA), anti-claudin-4 (diluted 1:100, mouse

monoclonal, Zymed Inc., San Francisco, CA, USA), anti-claudin-7 (diluted 1:80, rabbit polyclonal, Zymed Inc., San Francisco, CA, USA) and anti-E-cadherin (diluted 1:50, mouse monoclonal, DAKO), respectively for 60 min at room temperature. Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO, LSAB2 Kit). The chromogen substrate was 3, 3-diaminobenzidine in each case (DAB substrate-chromogen, DAKO). Sections were counterstained with Mayer's haematoxylin. A negative control was performed by omission of the primary antibody. For each claudin as positive control appropriate tissue blocks were used (**Table 2**) (Jakab et al. 2008). In each case, two independent observers recorded the distribution of staining, intensity and localization.

Table 2 The positive controls of claudin-1, -4, and -7, E-cadherin.

	Provider	**Concentration**	**Positive control tissue**	**Staining pattern**
Claudin-1	*Zymed* (polyclonal rabbit antibody)	1:100	Normal canine skin (epithelial cells of the stratum spinosum, apocrinocytes and sebocytes)	Membrane
Claudin-4	*Zymed* (monoclonal mouse antibody)	1:100	Normal canine skin (apocrin gland cells)	Membrane
Claudin-7	*Zymed* (polyclonal rabbit antibody)	1:80	Normal canine skin (apocrin gland cells)	Membrane
E-cadherin	*DAKO* (monoclonal mouse antibody)	1:50	Epithelial cells of apocrin glands	Membrane

2.8. Investigation of PKC isoenzymes using Western blot

Hydrogen peroxide-treated (1 mM hydrogen peroxide, 1 h) cells and untreated controls (each from a 10 cm Petri dish) were washed twice with 10 ml pre-chilled PBS. For lysis cells were resuspended in (500 µl/10 cm tissue culture plate) ice-cold ZET buffer (50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1% Triton X-100), supplemented with 1x proteases inhibitor and phosphatases inhibitor cocktails (Roche Applied Science, UK). The lysed cell suspension was incubated on ice for 1 h and centrifuged at 10 000 g for 30 min at 4°C. After transfer of the supernatants into fresh Eppendorf tubes, protein concentrations were

determined according to Bradford (1976). Equal amounts of proteins from the extracts were loaded onto 8-16% HEPES-SDS-polyacrylamide gels (Pierce, Rockford, IL, USA). Gels were blotted to Immobilon-P membranes (Millipore GmbH, Schwalbach, Germany) by a standard procedure. For immunodetection, membranes were first blocked for 1 h with 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween 20 in Tris-buffered saline (pH 7.5), at room temperature, and then treated with the antibodies and processed according to manufacturers' instructions. The following antibodies were used: rabbit polyclonal antibodies against PKCα, PKCγ, PKCδ, PKCε, PKCζ and PKCζ-Thr[410/403] (all from Cell Signaling Technology, Danvers, MA, USA) and mouse monoclonal antibody against GAPDH (Millipore). PKCγ was detected with an antibody (C-19, Santa Cruz Biotechnology) mapping with an epitope of 15-25 amino acids within the last 50 amino acids at the C-terminus of PKCγ. Secondary antibodies conjugated with horseradish peroxidase were: goat anti-rabbit IgG, goat anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK) and donkey anti-goat IgG (Santa Cruz Biotechnology). Visualization of blots was performed with enhanced chemiluminescence by Immobilon Western (Millipore), and scanning with GelImager (INTAS, Göttingen, Germany).

To examine the extent of hydrogen peroxide-induced upregulation of PKCζ, treatments with 1 mM hydrogen peroxide for 1 h were performed.

2.9. Lipid peroxidation in IPEC-J2 cells exposed to peroxide treatment

The rate of lipid peroxidation was determined by quantitative measurement of conjugated dienes (CDs), conjugated trienes (CTs) and malondialdehyde (MDA).

The peroxide concentration was 1, 2 and 4 mM and treatment was for 1 h. After washing the cells with PBS twice, 1.5 ml 0.3 w/v % potassium chloride solution was applied and cells were scratched off from the culture dish with a rubber policeman and transferred to centrifuge tubes. Cell suspension was sonicated for 20 sec and centrifuged at 3000 g for 20 min at 5°C. Cell supernatants were used for the spectrophotometric analysis of lipid peroxidation markers such as CD at 234 nm, CT at 268 nm and MDA at 535 nm detection wavelength. To evaluate the status of cellular antioxidant defence, reduced glutathione content was determined by the 5,5'-ditiobis (2-nitrobenzoic acid) reaction (Sedlak and Lindsay 1968) and glutathione peroxidase activity with end-point direct assay in the presence of cumene-hydroperoxide and reduced glutathione (Lawrence and Burk 1976) was spectrophotometrically measured.

Conjugated dienes and conjugated trienes determined by a standard procedure (AOCS 1998).

For MDA measurement, 1.125 ml of mixture of 10% perchloric acid and 0.76% tiobarbituric acid (TBA) solution and 10% trichloroacetic acid (TCA) was pipetted into 0.25 ml sample followed by 20 min incubation at 100°C. The absorbance of the supernatants was determined at 535 nm against reagent blanks after cooling and centrifuging the samples at 3000 g for 15 min at 4° C according to Botsoglou et al (1994). Using the molar extinction coefficient of the MDA–TBA complex of $1.49X\ 10^5\ M^{-1}\ cm^{-1}$ [Draper and Hadley, 1990], the amount of TBARS was expressed as nmol MDA equivalents formed per mg cell protein measured in a further set of control cultures not used for TBA assay.

2.10. Quantitative Real Time PCR

After the H_2O_2 treatment 1 ml of ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to the cell mono-layers. Samples were collected and kept at -80°C until further process. Total RNA was isolated from the cells according to the manufacturer's instructions. To prevent DNA contamination, the isolated RNA (2 μg) was treated with the AMP-D1 DNase I (Sigma). Integrity of the isolated RNA was examined by electrophoresis. The resulting bands of the 18S and 28S rRNA were visualised and scanned by the InGenius LHR Gel Documentation and Analysis System (Syngene, Cambridge, UK). Quantity, A_{260}/A_{280} and A_{260}/A_{230} ratios of the extracted RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Quality and quantity control of the isolated RNA was carried out both before and after the DNase treatment.

Synthesis of the first strand of cDNA from 1000 ng of total RNA was achieved using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's recommendations, using the random hexamer as a priming method. Quantitative real time PCR (qRT-PCR) was performed using the iQ SYBR Green Supermix kit (BioRad, Hercules, CA, USA) on the MiniOpticon System (BioRad). The cDNA was diluted 5-fold before equal amounts were added to duplicate qRT-PCR reactions. Tested genes of interests were interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α) and heat shock protein 70 (Hsp 70). Reference genes were hypoxanthine phosphoribosyl-transferase (HPRT) and cyclophilin-A (CycA). Sequences of the tested genes are listed in **Table 3**. For each PCR reaction, 2.5 μl cDNA was added directly to a PCR reaction mixture, set to a final volume of 25 μl, containing 1x concentrated iQ SYBR Green Supermix and 0.2 μM of the appropriate primers. The thermal profile for all reactions was 3

min at 95°C, 40 cycles of 20 sec 95°C, 30 sec at 60°C, and 30 sec at 72°C. At the end of each cycle, the fluorescence monitoring was for 10 sec. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. In order to determine the efficiencies of the PCR reactions standard curves were obtained for each target and reference genes using serial dilutions of a reference cDNA. Real-time PCR efficiencies (E) were calculated according to the equation: $E=10^{(-1/slope)}$. To determine the stability of the reference genes, the geNorm (version 3.5) was used. The PCR amplicons were separated by electrophoresis in 2 % agarose gel, the resulting bands visualised and scanned by the InGenius LHR Gel Documentation and Analysis System and quantified by the GeneTools Software (Syngene).

Table 3 Sequence of primer sets used for quantitative real-time PCR (F: forward, R: reverse)

Gene symbol	Accession number	Primer sequences	Product size(bp)	Efficiency	R^2	Reference
IL-6	NM_214399	F 5'-TTCACCTCTCCGGACAAAAC-3'	122	1.970	0.995	Sakumoto et al., 2006
		R 5'-TCTGCCAGTACCTCCTTGCT-3'				
IL-8	NM_213867	F 5'-AGAGGTCTGCCTGGACCCCA-3'	126	1.972	0.999	Paszti-Gere, 2012a
		R 5'-GGGAGCCACGGAGAATGGGT-3'				
TNF-α	NM_214022	F 5'-TTCCAGCTGGCCCCTTGAGC-3'	146	1.873	0.982	Hyland et al., 2006
		R 5'-GAGGGCATTGGCATACCCAC-3'				
Hsp 70	NM_001123127	F 5'-GCCCTGAATCCGCAGAATA-3'	152	1.95	0.991	Paszti-Gere, 2012b
		R 5'-TCCCCACGGTAGGAAACG-3'				
HPRT	NM_001032376	F 5'-GGACTTGAATCATGTTTGTG-3'	91	1.963	0.997	Nygard et al., 2007
		R 5'-CAGATGTTTCCAAACTCAAC-3'				
CycA	NM_214353	F 5'-GCGTCTCCTTCGAGCTGTT-3'	160	1.907	0.998	Hyland et al., 2006
		R 5'-CCATTATGGCGTGTGAAGTC-3'				

2.11. IL-8 ELISA

After treatment with 1 mM H_2O_2 for 1 h, Caco-2H and IPEC-J2 cells were incubated for 0, 1, 4, 6 and 24 hs. The apical and basolateral culture media were collected and the level of IL-8 secretion by IPEC-J2 cells was determined by a porcine-specific IL-8 ELISA Kit (Invitrogen). In culture media of Caco-2 cells, IL-8 concentrations were assayed using the IL-8 Cytosets TM antibody pair kit containing matched, pre-filtered and fully optimized capture and detection antibodies, recombinant standard and streptavidin- horseradish peroxidase (Biosource Europe S. A., Nivelles, Belgium).

2.12. Effect of SCSs on IPEC-J2 cells exposed to oxidative stress

L. plantarum 2142 (Culture Collection of the Institute of Dairy Microbiology, Agricultural Faculty of Perugia University, Perugia, Italy), *L. casei* Shirota (Culture Collection of University Utrecht, Department of Pathology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands), *Enterococcus faecium* CECT 4515 (Norel Animal Nutrition, Madrid, Spain) were grown in DeMan, Rogosa, Sharpe (MRS) broth. For propagation of *Bacillus amyloliquefaciens* CECT 5940 tryptone soya broth (TSB) was used, *Bifidobacterium animalis* subsp. *lactis* Bb 12 (Chr. Hansen Holding A/S, Horsholm, Denmark) was cultured in tryptone phytone yeast (TPY*)* medium.

Inoculation was accomplished with a stationary culture of a probiotical strain (1% inoculum). The bacteria were grown for 24 h at 37 °C and subcultured at least twice prior to the experiments. Spent culture supernatants (SCSs) were prepared by centrifugation of the bacterial suspension (with final bacterial concentrations of 10^9 CFU/ ml) at 3000 g at 5°C for 10 min. Centrifuged culture supernatant was then passed through a sterile 0.22 μm pore size filter unit.

Before treatments confluent monolayers of IPEC-J2 cells were washed twice with complete medium without antibiotics and fetal bovine serum (plain medium). Cells were treated apically with 1 mM hydrogen peroxide in a plain medium for 1 h. The hydrogen peroxide solution was prepared freshly prior to each experiment. To monitor the potential beneficial effect of selected probiotics, IPEC-J2 cells were treated with certain SCS simultaneously with hydrogen peroxide. In controls, SCSs were replaced with bacterial growth media. After the incubation period, cells were washed twice with plain medium and were subjected to RNA extraction. TER measurements were performed both before and after the hydrogen peroxide treatment.

2.13. Determination of D- and L-lactic acid (LA) produced by lactobacilli

Enzymatic analysis of D-and L- LA was performed to ascertain the concentration of the LA present in SCS of lactobacilli with a lactate dehydrogenase-based UV detection kit (Enzymatic BioAnalysis, R-Biopharm AG, Germany). The assay principle was the oxidation of D-lactate or L-lactate to pyruvate in the presence of appropriate enantiomer-selective lactate dehydrogenase, with the amount of NADH formed being stoichiometric to the amount of D-LA or L-LA. The increase in NADH was determined as absorbance increase at 340 nm.

2.14. Treatment of IPEC-J2 cells with carboxylic acids

Acetic acid and racemic, D-and L-LA were obtained from Sigma-Aldrich Co. In the experiments acetic acid was used at 10 and 50 mM concentrations, D- LA at 100 mM and L-LA at 65 mM in phenol red-free plain DMEM. Cells were treated apically with the selected organic acid in a plain medium for 1 h in the presence and in the absence of peroxide. After the incubation period, cells were washed twice with plain medium and were subjected to RNA extraction procedure. TER measurements were performed both before and after the organic acid acid application. The pH values of the solutions were measured with a microprocessor pH meter (pH 3000 WRW, Weilheim Germany).

2.15. Separation of L. plantarum 2142–specific bioactive peptides

Capillary zone electrophoresis (Bio-Rad Laboratories, Inc., BioFocus 2000) was applied for separation of underivatized peptides with UV detection at 200 nm wavelength using 0.1 M phosphate buffer. The temperature of uncoated glass capillary was adjusted at 38°C. Prior to electrophorectic run, the sample was heat-treated at 100°C for 10 min to remove macromolecules from the solution. The method was capable of detection of the reference dipeptide carnosine (β-alanyl-L-histidine) at a concentration as low as 0.016 μM.

2.16. SDS-PAGE profile of probiotic proteins

Gel electrophoresis was performed with a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Inc.) instrument using 15% acrylamide gels according to Laemmli. For sample preparation 10 mg freeze-dried sample was dissolved in 300 μl buffer (3% sodium dodecyl sulfate, 62 mM tris (hydroxymethyl) aminomethane, 8.7% glycerol, 10% β-mercaptoethanol pH=6.8). Components of running buffer were 25mM Tris-HCl, 200mM glycine and 0.1w/v% SDS. The solution was boiled for 5 min, then 5, 10 and 15 μl sample were added to gel wells. Gel staining was performed with the application of Coomassie R-250 solution. The stained gel was photographed on a UV transillumination table with the Gel Documentation System 2000 and subsequent densitometric analysis of the gel was with Quantity One 4.3.0 software package (Bio-Rad Laboratories, Inc.) comparing sample proteins with those of M_r marker LMW mixture (Amersham Pharmacia Biotech Inc.).

2.17. Butyrate effects in vitro and in vivo on swine ileum

The antimicrobial effect of sodium-n-butyrate (4 mM- 100 mM) was investigated on growth of *Lactobacillus plantarum* 2142 and *Escherichia coli* 30037. DeMan Rogosa Sharpe (MRS) and Müller-Hinton broth (pH=6) were used as a growth media for *Lactobacillus plantarum* 2142 and for *E. coli* 30037, respectively. Bacterial suspension without sodium-n-butyrate in the broth served as control. The low pH and higher acetate concentration facilitated the selective growth of *lactobacilli* based on visual colony counting. The incubation was performed at 37°C under aerobic conditions during continuous stirring at 200 g. The optical density was read 0-24 hs after inoculation at 630 nm wavelength.

For *in vivo* research a total of 40 (mixed male and female) commercial cross pigs (Hungarian Large White x Hungarian Landrace /HLW x HL/) were used. The experiments were performed at the Experimental Unit of the Research Institute for Animal Breeding and Nutrition Herceghalom, Hungary. The treatment, housing, husbandry and slaughter conditions were set to meet the requirements of European Union Guidelines. Pigs were free of the principal swine infectious agents at the beginning and at the end of the study. The animals were placed in individual cages and they were allowed *ad libitum* access to feed and water. Growth performance and feed intake were monitored weekly. Average temperature was 20°C and relative humidity was 80% during the experiment. The composition of feed is summarized in **Table 4**. The experimental conditions were kept unaltered, the only difference was that diet supplemented with sodium-n-butyrate (0.15%, 13.6 mM) was administered to treated animals, while butyrate-free feed was added to controls. The feed of swine with premix containing sodium-n-butyrate lasted for 28 days. The animals were slaughtered at the age of 115 days under permission in the Research Institute for Animal Breeding and Nutrition (Herceghalom). Butyrate content of the samples and that of the feed without antibiotics and growth promoters were determined by gas chromatography. Immunohistochemical measurements for evaluation of the number of dividing cells in ileal samples were performed after dehyration, embedding in paraffin and section cutting using Ki67 proliferation marker. The count of *lactobacilli* in swine ileum was determined on MRS broth based on visual colony inspection (20-150 colonies per Petri plate). The incubation lasted at 37°C for 72 h. Analysis of lactic acid enantiomers (D- and L-lactate) from ileal samples was performed using LDH-based UV test (Enzymatic BioAnalysis, R-Biopharm AG, Germany *Section 2.13*).

Table 4 Composition of the swine feed (%)

Components	Feed	
	Experimental	Control
Supplementary ingredient /sodium-n-butyrate/	**0.15**	
Zeolite universal		**0.15**
Corn	57.91	57.91
Soybean meal 46%	16.97	16.97
Extracted sunflower meal 37%	10.00	10.00
Maize	11.37	11.37
Feed lime	1.30	1.30
MCP	1.10	1.10
Sodium chloride	0.50	0.50
L-lysine-HCl	0.20	0.20
Swine complete premix*	0.50	0.50

*Premix composition (1000 g contains): Vitamin A (E672) 2055863 IU; Vitamin D_3 (E671) 400000 IU; Vitamin E (α-tocopherol) 4263 mg; Vitamin K_3, 102 mg; Vitamin B_1 72 mg; Vitamin B_2; 269 mg; Vitamin B_6; 168 mg; Vitamin B_{12}; 1.88 mg; Panthothenic acid 794 mg; Folic acid 46 mg; Biotin 4.8 mg; Niacin 4409 mg; Choline chloride 72100 mg; Vitamin C 1140 mg; Fe 22400 mg; Mn 16880 mg; Cu 2800 mg; Zn 20000 mg; Se 84 mg; Co 103 mg; I 120 mg.

2.18. Gentamicin transport through IPEC-J2 cell monolayer

For the preparation of the apical medium, the gentamicin sulphate stock solution (50 mg/ml, Sigma, Germany) was 50* diluted with plain DMEM/F12 medium. Six-six wells in 6-well plates were used for testing the penetration of gentamicin across cells in the presence and absence of 0.5-1 mM H_2O_2. PBS was also applied as a replacement of DMEM/F12 to test if the lack of Ca^{2+} ions can affect monolayer resistance. To supply appropriate amounts of medium for HPLC analysis, two different plates were used for 1 h and 6 h determinations. In the basolateral compartment simple DMEM medium or where it is indicated PBS was used. For HPLC measurements acetonitrile (Prolabo, HiPerSolv isocratic grade), KOH, boric acid, fluorenylmethyloxycarbonyl chloride (FMOC), glycine (Merck, ar) were used. During derivatization 800 µl prepared sample was pipetted into a Merck vial, 400 µl of FMOC was added and vortexed for 30 sec. Sample was kept at room temperature for 15 minutes. Reaction was stopped with 40 µl glycine solution and vortexed for 30 sec. Limit of detection (LOD) was 0.19 ng/ml, limit of quantification (LOQ) was 0.63 ng/ml indicating a high sensitivity of the method.

For preparation of a calibration curve 50 mg/ml gentamicin was diluted with 50% acetonitrile and 50 % borate buffer (pH=8.5) and standard gentamicin sulphate concentration range was 0.1-2 µg/ml (r^2> 0.99). For HPLC separation (Merck 1 HPLC system) the following conditions were selected: The eluent composition was 85% acetonitrile and 15%

ultrapure water (UPW), the HPLC column was Spherisorb ODS-2 5 μm 250x 4,6 mm, the temperature of the column 25°C and that for the autosampler 20 °C. 25 μl sample was injected, running time was 34 min with 1 ml/min flow rate. The fluorescent excitation detection wavelength was 260 nm, and emission wavelength was 315 nm for derivatized gentamicin.

2.19. Statistical analysis

Relative gene expression levels of the genes of interest were calculated by the Relative Expression Software Tool (REST) 2009 Software. The data are given as mean values ± S.E.M (n) where n refers to the number of parallel measurements. For statistical evaluation R 2.11.1 software package (2010) was applied. Differences between means were evaluated by one-way analysis of variance (one-way ANOVA) with post-hoc Tukey test, where data were of normal distribution and homogeneity of variances was confirmed or Kruskal-Wallis nonparametric test. Analysis of covariance (ANCOVA) method was used for interpretation of change in TER versus culturing time in case of different membrane inserts. A p value of < 0.05 was accepted to indicate statistical significance.

3. Results

3.1. Applicability of in vitro models for studying the effects of intestinal oxidative stress

3.1.1. Heterogeneity of Caco-2 cell line

Preliminary study was performed to establish whether Caco-2H and P cells are the most suitable *in vitro* system to investigate acute oxidative stress-induced (sub)cellular changes. TER values were measured in the absence of peroxide treatment (**Fig. 10**). We established that more than 10-fold differences in TERs can be observed in 25 day follow-up period between transepithelial resistance values of Caco-2H versus Caco-2P cells. The degree of differentiation was remarkably low in Caco-2H cells under the same culturing conditions, while Caco-2P cells achieved TER values as high as ~6000 Ohm*cm^2.
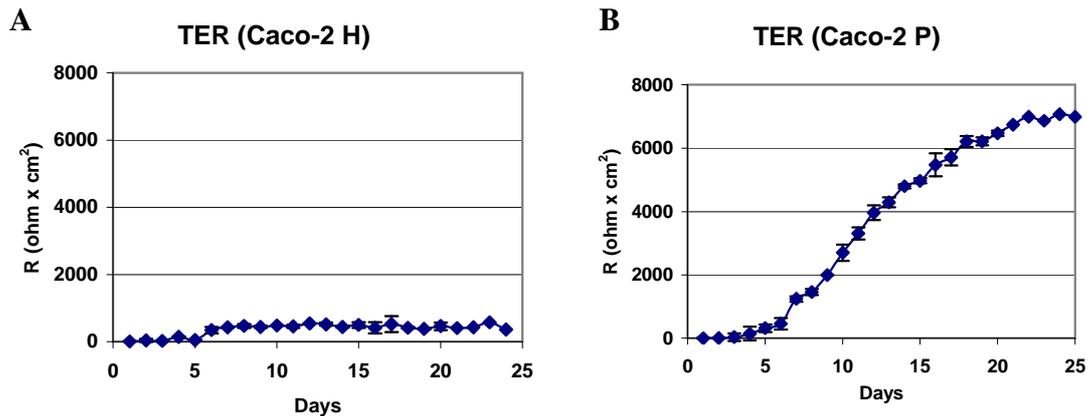
Fig. 10 Comparisons of differentiation rate for Caco-2H (A) and Caco-2P (B). Caco-2P cells were highly polarized, TER values reached 6000 Ohm*cm^2 within 20 days in contrast to Caco-2H cells, where the electrical resistance remained below 1000 Ohm*cm^2 over 25 days of culturing time. The mean values $\pm$ SEM are expressed in Ohm*cm^2 (n=5).

To assess a possible difference in peroxide sensitivity exists between Caco-2H and Caco-2P cells, they were treated with 1 mM hydrogen peroxide for an 1 h and the cell death rate was investigated by DAPI. Early effect of hydrogen peroxide was also monitored. Cell membranes of filter-grown Caco-2P did not become permeable when they were exposed less than 10 mM H_2O_2 in contrast to the less polarized Caco-2H, which was extremely sensitive to peroxide-induced oxidative stress (**Fig. 11**).
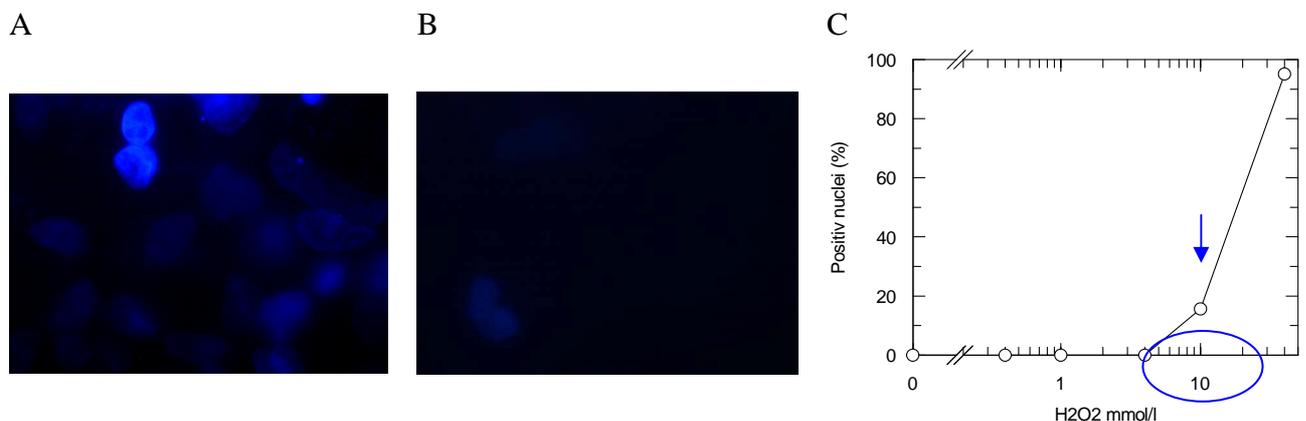
Fig. 11 Differential cell death sensitivity to oxidative stress in two Caco-2 cell lines of different origins. Caco-2H (A) was more sensitive to a 1 h 1 mM H_2O_2 treatment compared to Caco-2P (B), where cell death was not detected under these conditions. The percentage of dead cells based on proportion of DAPI-marked positive nuclei (C) became prominent only when more than 10 mM peroxide was applied to Caco-2P cells for 1 h.

3.1.2. The effect of oxidative stress on IL-8 expression

The change in gene expression levels of inflammatory cytokines and Hsp 70 determined by qRT-PCR was a good indicator for evaluation how the acute oxidative stress influences cell responses in short period of time and which metabolites of probiotics can exert quenching effect on unfavourable cytokine upregulation. The data have shown that the level of proinflammatory cytokines increased significantly by the effect of hydrogen peroxide administration. Production of IL-8 quantified by ELISA pointed out the time shift between gene transcription and protein synthesis. Concentration of IL-8 was monitored in both compartments, the apical and basolateral side of IPEC-J2 cells exposed to 1 h 1 mM H_2O_2 treatment.
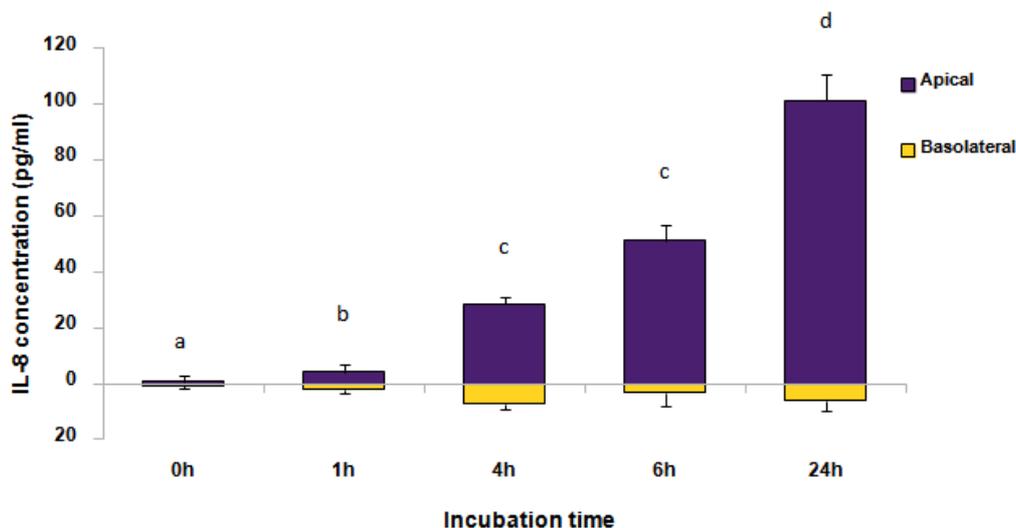
Fig. 12 After 0-24 h incubation IL-8 contents in apical and basolateral medium of IPEC-J2 cells under acute oxidative stress (1 mM H_2O_2). Significant differences between groups are indicated with different letters (p<0.05). Average± S. E. M (n=7)

Significant increase in IL-8 concentration in apical compartment as early as 4 h after peroxide administration can be seen compared to low basal secretion without ROS in **Fig. 12**. Time dependence of increase in IL-8 synthesis demonstrated as the cytokine amount almost doubled after 6 h incubation compared to that at 4 h with the peak level at 24 h after H_2O_2 treatment (100X compared to control values). In contrast to peak IL-8 gene expression at 1 h after hydrogen peroxide introduction protein synthesis occurred at slower rate with the final value of 100 pg/ml 1 d after ROS exposure. IL-8 production was not significantly associated with basal secretion demonstrating that the passage of cytokines into apical compartments

mimicks the *in vivo* situation where cytokine trafficking occurs from enterocytes into the luminal space.

Media from the apical and basolateral compartments of Caco-2H cells were also collected and assayed for IL-8 at 0, 1, 4, 6 and 24 hs after 1 mM peroxide addition for an h. On **Fig. 13** significant increase in IL-8 production of Caco-2H cells was detected in medium from apical region as early as at 1 hr sampling point of recovery time. Exposure of Caco-2H cells to 1 mM H_2O_2 led to a significant increase in IL-8 amount during recovery reaching the highest value 456, 6±15,6 pg/ml at 24 h proving the rapid and pronounced reactivity of Caco-2H cells to acute oxidative stress in respect to elevated IL-8 production.
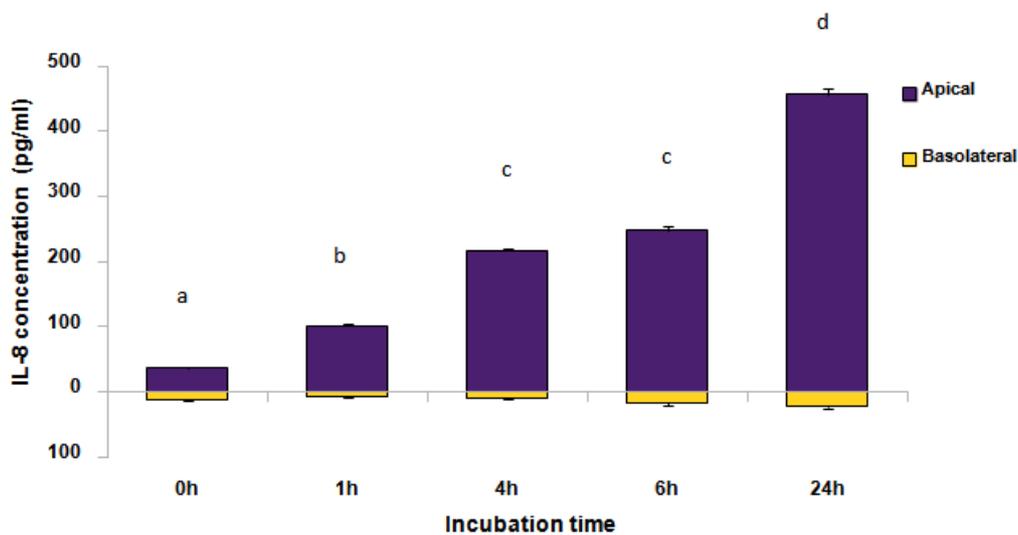
Fig. 13 IL-8 protein production of Caco-2H cells after a 1 h treatment with 1 mM H_2O_2. Zero time is after removal of hydrogen peroxide, when cells were allowed to recover in plain DMEM for up to 24 h. In the supernatants IL-8 levels were determined by ELISA. Means and SEMs of triplicates are given. Significant increases in IL-8 amounts compared to controls are shown with different letters (p<0.05).

3.1.3. ROS-triggered transepithelial resistance changes

To asses the effect of H_2O_2 on the integrity of cell monolayer TER was determined before and after H_2O_2 treatment. TER values did not differ significantly in case of Caco-2H cells over 0-4 mM peroxide concentration range indicating that barrier function of the membrane remained intact. However, 40 mM hydrogen peroxide for 1 h caused a marked loss as mediated by 22% of the initial control TER value was detected. There was no significant difference in TER change between control, 0.5 mM and 1 mM H_2O_2-treated differentiated IPEC-J2 cells. TER decreased significantly in polarized IPEC-J2 cells exposed to 2 mM and 4

mM H_2O_2 compared to the lower-dose treatments. In contrast, in Caco-2H cells exposed to peroxide at 4 mM no significant differences among TER values of control and treated groups could be observed (**Fig. 14**).
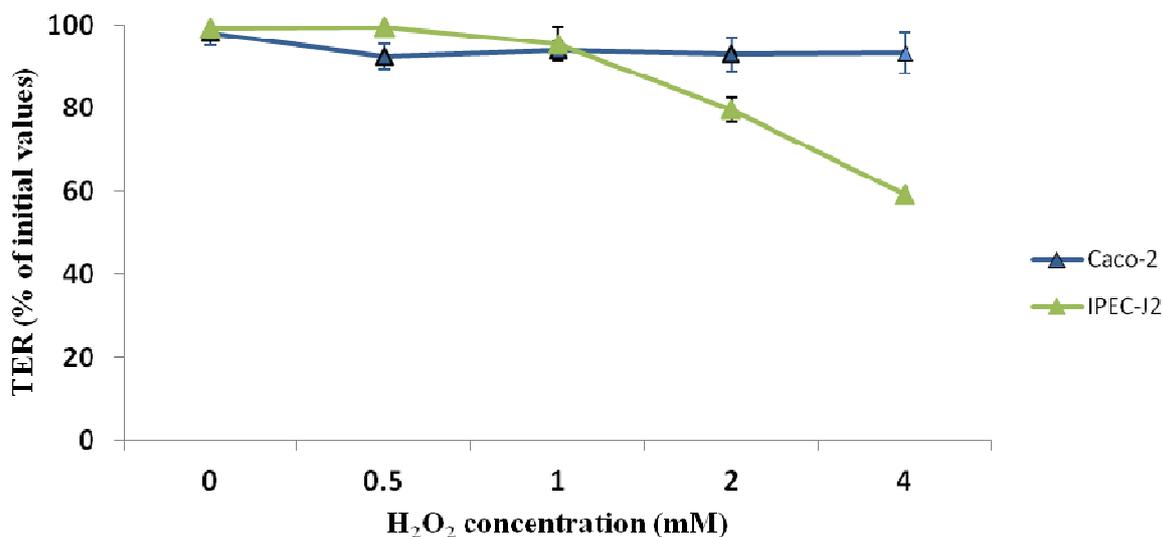
Fig. 14 Effect of H_2O_2 exposure on the integrity of Caco-2P and IPEC-J2 cells grown on membrane inserts as determined by the decrease of the TER. Higher sensitivity of IPEC-J2 cells to oxidative stress compared to Caco-2P cells is demonstrated based on significant reduction in the resistance (p< 0.05). Values are means ± SEM (n=6 for TER).

3.2. Optimizing IPEC-J2 culturing conditions: Polycarbonate vs cc-PE membrane inserts

Functional integrity of monolayers of IPEC-J2 cells was determined by TER measurements. Cells were grown on Transwell polycarbonate and collagen-coated Transwell polyester membrane inserts for up to 21 days, and TER was measured on alternate days. Significant difference was not found between the degree of IPEC-J2 cell differentiation on PC and cc-PE membrane inserts till the 8th day of cell culture. Linear regression of TER values was used (between the 9th and 19th day of cell culture) to evaluate functional integrity of cell monolayer on each filter. During this period the increase in TER of IPEC-J2 cells grown on cc-PE membrane inserts was significantly higher (slope=802.7±24.2 Ohm*cm^2/d, R=0.9608) than that on PC inserts (slope=663.0±37.5, Ohm*cm^2/d R=0.8789). On the 21st day of cell culture the final TER values were significantly higher on cc-PE (8702.8±45.9) than on PC (6134.8±154.3) membrane inserts (p<0.05) (**Fig. 15**). For this reason, the cc-PE membrane inserts were used in the present study. Cell monolayers were allowed to reach TER values of ≥ 8000 Ohm*cm^2, which was routinely after 17 days.
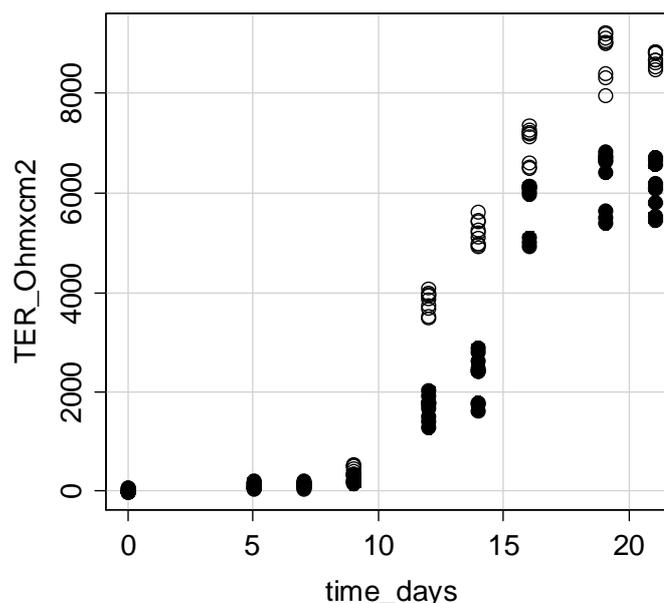
Fig. 15 TER of IPEC-J2 mono-layers for 20 days cultured on Transwell polycarbonate (PC; filled circles, n=3) and collagen-coated Transwell polyester (cc-PE; open circles, n=3) membrane inserts. Each circle represents measurements at single membrane inserts. Cells were seeded at a density of 1.5×10^5 per well on both membrane inserts (pore size 0.4 μm; surface area 4.67 cm^2). Between the 9th and 19th day of cell culturing TER increased linearly over time (slope $_{PC}$=663.0 Ohm*cm^2/d, R_{PC}=0.8789; slope $_{cc-PE}$=802.7 Ohm*cm^2/d, R_{cc-PE}=0.9608). On the 21st day of culture the final TER values were significantly greater on cc-PE (8702.8±45.9) than on PC (6134.8±154.3) membrane inserts (p<0.05).

3.3. Dose and post-treatment time-course dependencies of H_2O_2-induced cytokine mRNA expression

H_2O_2 treatment in the concentration range 0.5-4 mM for 1 h did not alter the relative gene expression level of IL-6 in IPEC-J2 cells. However, mRNA expression level of IL-8 was elevated (p<0.05) after treatment with 0.5, 1 and 2 mM H_2O_2 administration compared to controls with the peak level reached at 1 mM H_2O_2. TNF-α gene expression was significantly up-regulated compared to the control (p<0.05) after 0.5, 1, 2 and 4 mM H_2O_2 administration, with the highest level at 1 mM H_2O_2. At higher hydrogen peroxide concentrations gene expression levels of both IL-8 (from 2 mM) and TNF-α (from 4 mM) declined significantly (n=4) (**Fig. 16**).
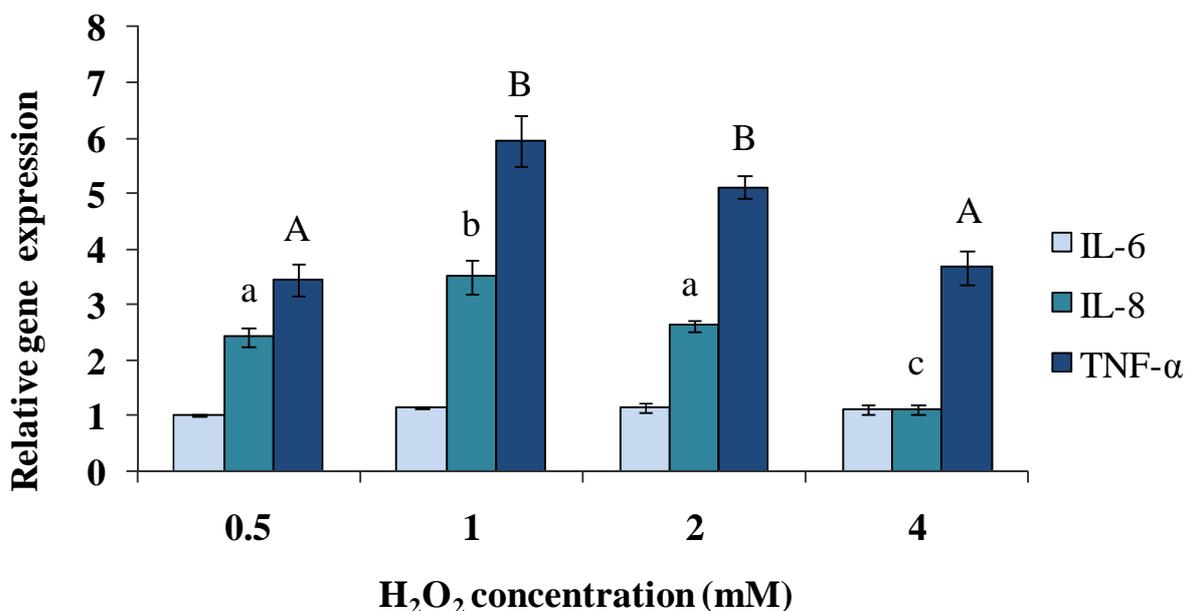
Fig. 16 Relative gene expression levels (qRT-PCR) of IL-6, IL-8 and TNF-α in IPEC-J2 cells grown on collagen-coated Transwell polyester membrane inserts and exposed to the given H_2O_2 concentrations for 1 h. Relative gene expression levels of inflammatory cytokines are described as means ± SEM (n=4). IL-8 series are indicated with small letters, TNF-α series with capitals, columns labelled with c represent relative gene expression levels, which do not differ significantly from control. Different letters visualize pairwise statistical differences obtained from Tukey post-hoc test in each cytokine group (p<0.05). Relative gene expression of IL-6 did not change significantly in IPEC-J2 cells exposed to 1 h 0.5-4 mM H_2O_2 treatment (p>0.05).

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In the following experiments the oxidative stress-induced H_2O_2 was removed after 1 h and cytokine expression levels were determined by RT-PCR in the recovery time (0-24 h). Immediately after application of 1 mM H_2O_2 and 1 h thereafter, the relative gene expression levels of two investigated inflammatory cytokines (IL-8 and TNF-α) were elevated in IPEC-J2 cells (**Fig. 17**). As hydrogen peroxide entered the cells from the onset of H_2O_2 administration to the end of 1 h treatment, the gene expression levels of IL-8 and TNF-α increased gradually at every 15 min sampling point until the highest expression levels were reached simultaneously with the almost complete peroxide uptake by IPEC-J2 cells (after 1 h of incubation). The peak gene expression level of TNF-α was observed 1 h after H_2O_2 treatment, which then showed significant decrease reaching the control value 4 h after the H_2O_2 exposure. Subsequently, the gene expression level of TNF-α became down-regulated 4-fold (6 h) and 6.25-fold (24 h). IL-8 gene expression level was significantly higher than control at 4 h and returned to control level at 6 h of H_2O_2 exposure. By the end of the recovery time (24 h) gene expression level became 3.5-fold down-regulated. No significant

48

change was observed in the expression level of IL-6 compared to control values between 0 and 24 h recovery time.
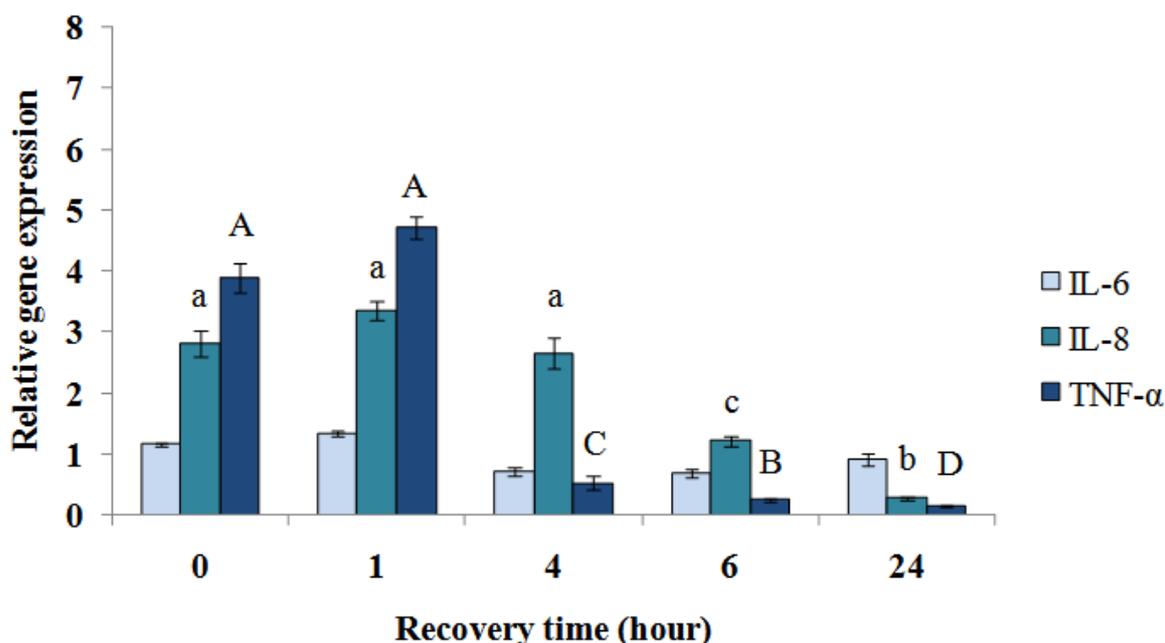
Fig. 17 Relative gene expression levels of IL-6, IL-8 and TNF-α mRNA in IPEC-J2 cells during recovery time. Cells were grown on collagen-coated Transwell polyester membrane inserts, and treated with 1 mM H_2O_2 for 1 h. Relative gene expression levels of the inflammatory cytokines are expressed as means ± SEM (n=6). Different letters show significant difference, small letters stand for IL-8 values, capital letters for TNF-α (p<0.05). Columns labelled with c and C represent relative gene expression levels, which do not differ significantly from control IL-8 and TNF-α values, respectively. Relative gene expression of IL-6 did not change significantly in IPEC-J2 cells exposed to 1h 1 mM H_2O_2 treatment during 24 h recovery time (p>0.05).

3.4. H_2O_2 degradation and consumption

Decomposition of H_2O_2 was monitored in distilled water and in plain medium without phenol red at 37°C up to 60 min (**Table 5**). Significant degradation of H_2O_2 was not observed in distilled water during the incubation time. However, in the plain medium the concentration of H_2O_2 decreased significantly. By the end of the incubation period (60 min), 21% of the H_2O_2 disappeared from the medium.

Table 5 Degradation of H_2O_2 in distilled water or plain medium without phenol red at 37°C

Incubation time (min)	H_2O_2 concentration (%)	
	Distilled water	Plain medium without phenol red
0	100[a]	100[a]
15	99.2 ± 0.76[a,1]	91.2 ± 1.24[ab]
30	98.1 ± 0.96[a]	85.9 ± 1.09[bc]
45	97.7 ± 0.55[a]	82.0 ± 1.63[c]
60	97.3 ± 0.61[a]	78.9 ± 2.02[d]

[1] Mean H_2O_2 concentration (%) $\pm$ SEM (n=3)
[a-d] Different superscripts indicate statistical differences (p<0.05)

To evaluate H_2O_2 consumption and transfer by IPEC-J2 cells extracellular H_2O_2 content was measured in the apical as well as basolateral media. After the 60 min incubation period less than 0.1% of the initial H_2O_2 was measured basolaterally. On the apical side cells took up 94% of the initial H_2O_2 during the 60 min incubation period (**Fig. 18).** Forty-three percent of the initial H_2O_2 amount was consumed in the first 15 min. Consumption of H_2O_2 followed a first-order kinetics with a rate constant (*k*) of $0.93 \pm 0.12 \times 10^{-3}$ s^{-1} (n=6). It has to be noted that degradation of H_2O_2 in plain medium could account for some of the decrease.
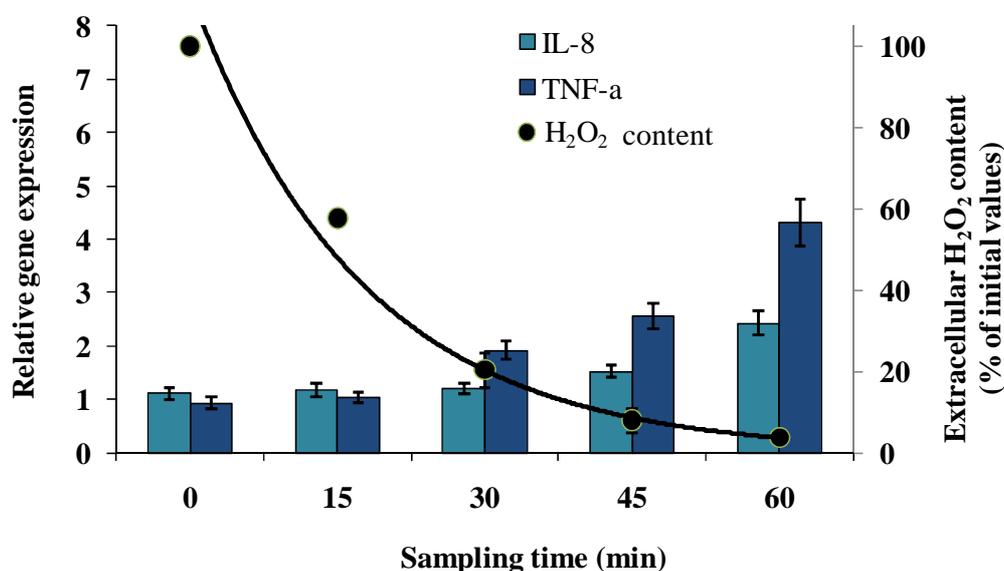
Fig. 18 Indirect time-course measurement of H_2O_2 consumption by IPEC-J2 cells grown on collagen-coated Transwell polyester membrane inserts, and the concomitant increase of IL-8 and TNF-α gene expression within the time of H_2O_2 exposure. Values indicate means $\pm$ SEM (n=6) Cells were treated with 1 mM H_2O_2 for 1 h apically and H_2O_2 concentration was determined every 15 minutes in the apical medium. H_2O_2 concentration in the apical medium is given as percentage of the initial concentration.

3.5. Lack of lipid peroxidation in IPEC-J2 cells exposed to millimolar peroxide

IPEC-J2 cells were treated with peroxide in the concentration range 1-4 mM for 1 h. An increase of lipid peroxidation was expected with increasing concentrations of H_2O_2 because with increasing oxidative stress at some point the antioxidant defense of enterocytes should be exhausted. However, this tendency could not be confirmed as indicators of lipid peroxidation such as MDA, CDs and CTs did not change significantly in IPEC-J2 cells under acute oxidative stress compared to basal values. Moreover, peroxide treatment did not affect the cellular level of reduced glutathione and the activity of scavenging enzyme, glutathione peroxidase significantly (**Table 6**).

Table 6 Capacity of glutathione defence system and early and late markers of lipid-peroxidation in IPEC-J2 cells. GSH: reduced glutathione, GSH-Px: glutation-peroxidase, MDA (malondialdehyde), CD (conjugated dienes), CT (conjugated trienes). Average ± SEM, n=3

	GSH (μM)	**GSH** (μM/g protein)	**GSH-Px** (U/g protein)	**MDA** (nmol/g protein)	**CD** (ABS 234 nm)	**CT** (ABS 268 nm)
0 mM H_2O_2	189.90 ± 2.3	161.21 ± 22.86	37.26 ± 5.69	2.50 ± 0.89	0.528 ± 0.18	0.122 ± 0.03
1 mM H_2O_2	198.19 ± 2.3	216.57 ± 38.84	44.00 ± 6.79	1.64 ± 0.35	0.577 ± 0.24	0.135 ± 0.04
2 mM H_2O_2	196.28 ± 2.3	181.72 ± 14.4	46.03 ± 3.52	1.85 ± 0.25	0.453 ± 0.03	0.107 ± 0.01
4 mM H_2O_2	235.15 ± 38.24	237.80 ± 57.17	42.14 ± 3.02	1.56 ± 0.33	0.790 ± 0.14	0.165 ± 0.3

3.6. DAPI-based determination of peroxide-triggered cell death

DAPI binds to the nuclei of the cells only after the plasma membrane becomes permeable (Nemeth et al. 2007a), therefore this method was applied to assay the level of cell death after H_2O_2 treatment at different concentrations. Extent of cell survival due to 0.5-1 mM H_2O_2 treatment was not statistically different from the untreated control. However, treatment with 2-4 mM H_2O_2 resulted in a moderate but significant increase in cell death of differentiated IPEC-J2 cells with 90% of the IPEC-J2 cells remained viable after 2 mM and 85% after a 4 mM H_2O_2 treatment (p<0.05) (**Fig. 19**).

0 mM H$_2$O$_2$ (Control)   **1 mM H$_2$O$_2$**   **4 mM H$_2$O$_2$**

ACDR= 2, 1±0.6%   **ACDR= 1,4±0.7%**   **ACDR= 14,6±2.8%**
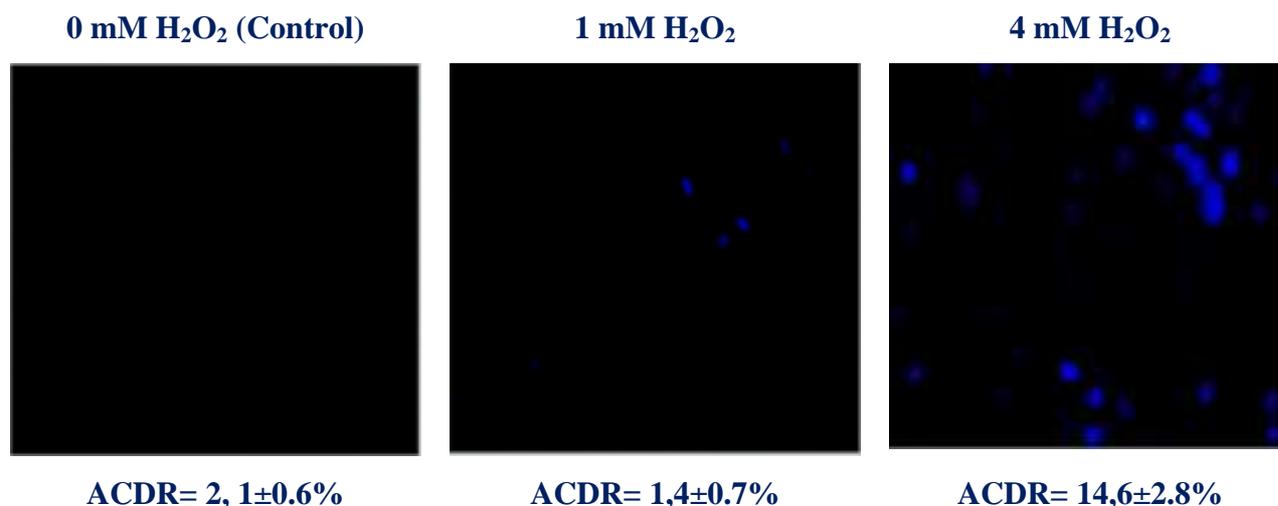
Fig. 19 Number of DAPI-positive nuclei correlates with the degree of cell death caused by peroxide administration in differentiated IPEC-J2 cells. The concentration of H$_2$O$_2$ varied between 0-4 mM. ACDR: average cell death degree based on the ratio of DAPI-stained to unstained nuclei counted from at least 10 microscopic fields.

3.7. Specific protein and peptide metabolites of Lactobacillus plantarum 2142

To evaluate the protein pattern of bioactive matrix yielded from *L. plantarum* 2142 secretion, comparisons of MRS broth (control) and SCS of *L. plantarum* 2142 (sample) were made after lyophilization using 15% SDS-PAGE. Parallel experiments (n=3) showed that two *L. plantarum* 2142 -specific proteins exist in the supernatant. A characteristic protein pattern can be seen in 20-29 kDa molecular weight range and another protein in smaller quantity between 29-37 kDa using Coomassie R-250 for detection. As indicated by the low molecular weight marker, the *L. plantarum* 2142-specific proteins had masses of 21 kDa and 31 kDa (**Fig. 20**).
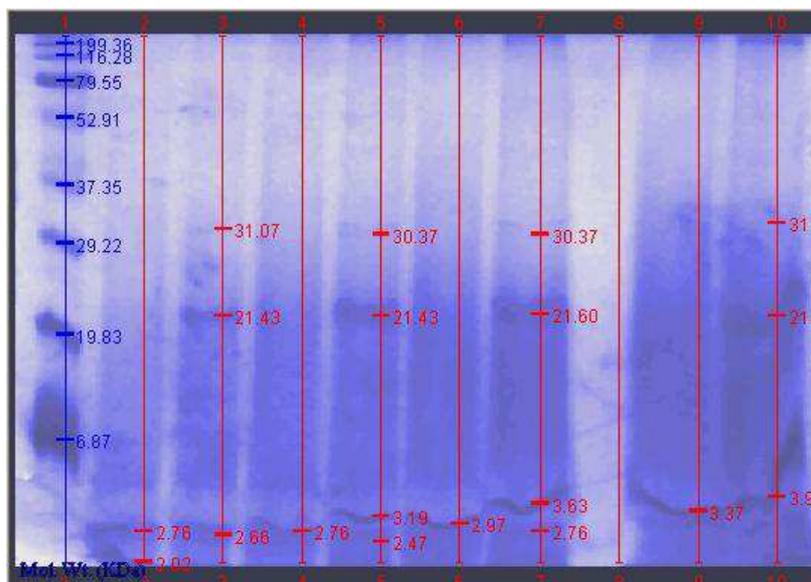
Fig. 20 Electropherogram of *L. plantarum* 2142 SCS proteins (lanes: 3rd, 5th, 7th) compared with MRS broth (lanes: 2nd, 4th, 6th) and low molecular weight marker (LMW first lane). Two characteristic differences in protein pattern were detected after separation by 15% SDS-PAGE using Coomassie R-250 for detection. With the aid of low molecular weight marker, the *L. plantarum* 2142-specific proteins were of 21 kDa and 31 kDa molecular weight. MRS broth was used as control. (n=3)

Peptides and proteins of lower molecular weight were analyzed by quantitative capillary zone electrophoresis. After substracting the electrophoretogram of MRS broth from SCS, a *L. plantarum* 2142-specific peptide of low-molecular weight was detected with the retention time 11.86 min (**Fig. 21).**
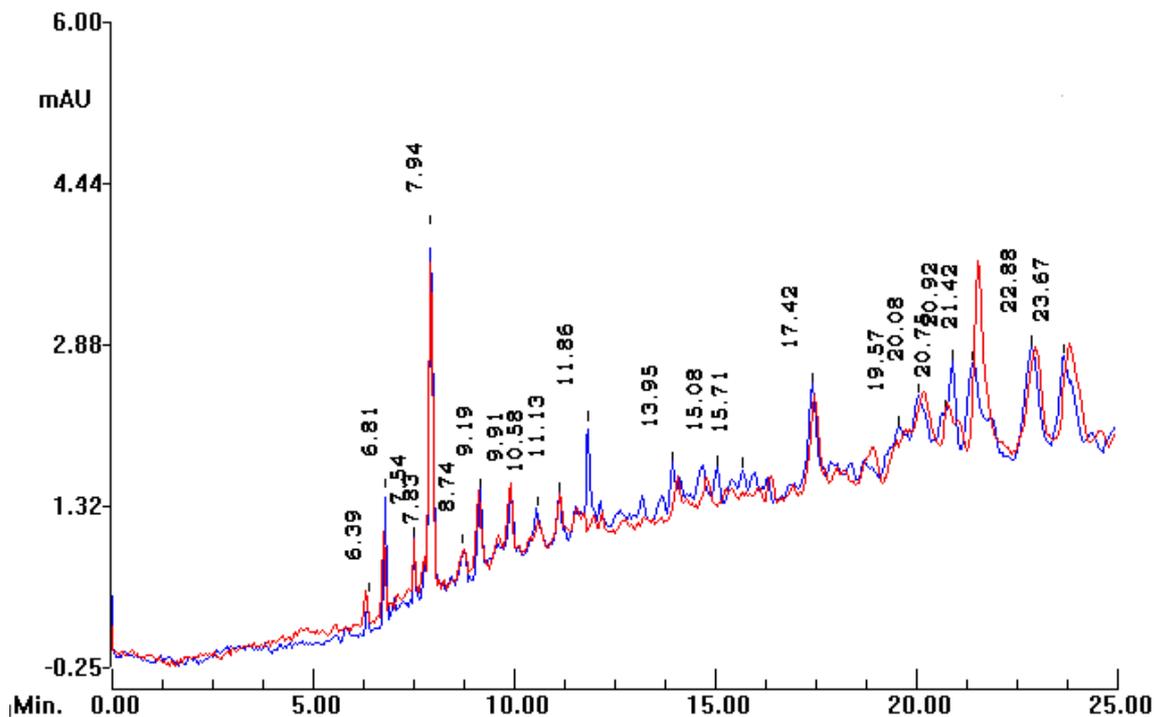
Fig. **21** Capillary electrophoretograms of heat-treated MRS (red line) and SCS of *L. plantarum* 2142 SCS (blue line). 0.1 M phosphate buffer was used as electrolyte and underivatized peptides were analyzed with UV detection at 200 nm wavelength. *L. plantarum* 2142-specific peptide was eluted from the uncoated capillary with 11.86 min retention time.

3.8. Gentamicin penetration through IPEC-J2 cell monolayer

There was no significant difference ($p > 0.05$) between TERs before the treatment and 1 or 6 h after treatment with gentamicin, thus it is assumed that cell integrity was not damaged by 1 mg/ml gentamicin (**Table 7**). Both immunohistochemical results and TER values suggested that at the given concentration gentamicin alone did not influence TJ formation and paracellular integrity (*data not shown*).

Table 7 TER values did not change significantly when IPEC-J2 cells were exposed to gentamicin at 1 mg/ml for 1 and 6 h (p> 0.05) compared to basal TERs in controls. Data are shown as mean± SD (n=3).

Treatment type	Treatment time (h)	TER before treatment (Ohm*cm^2)	TER after treatment (Ohm*cm^2)
1mg/ml gentamicin		9573.50	9835.02
1mg/ml gentamicin		9531.47	9690.25
1mg/ml gentamicin		9573.50	9704.26
MEAN		**9559.49**	**9743.18**
SD	1 h	**24.27**	**79.85**
1mg/ml gentamicin		8667.52	8616.15
1mg/ml gentamicin		8887.01	9083.15
1mg/ml gentamicin		9592.18	9732.28
MEAN		**9048.90**	**9143.86**
SD	6 h	**483.12**	**560.54**

Table 8 shows the transport of gentamicin in the presence and absence of 0.5 mM and 1 mM H_2O_2 through insert without cells and across the IPEC-J2 cell monolayer. In wells without cells, almost total equilibrium in gentamicin concentration was achieved after 6 h between apical and basolateral compartments. Addition of peroxide at different concentrations did not alter gentamicin transport significantly (p>0.05). The membrane insert served as a time-dependent barrier as it delayed the equilibration of gentamicin concentrations between the two compartments. In wells with cell monolayer, negligible amount of gentamicin was detected in the basolateral compartment both in wells treated with gentamicin and in wells treated with gentamicin + H_2O_2, for 1 h, and almost the total gentamicin content was detected in apical region (**Fig. 22**). Six h after treatment, a very low amount of gentamicin was measured in the basolateral compartment. No significant difference (p>0.05) was observed in gentamicin levels between wells with gentamicin alone, and those treated with gentamicin in combination with peroxide. Thus, up to 6 h of incubation gentamicin showed poor transcellular and paracellular permeation across a cell monolayer *in vitro* even when IPEC-J2 cell monolayer was incubated with millimolar peroxide.

Table 8 Concentrations of gentamicin (%) in the apical and basolateral compartments in wells containing IPEC-J2 cell monolayer and in control wells without cells. Peroxide was applied (concentrations were 0.5 and 1 mM H_2O_2 for 1 h in two-phased treatment (gentamicin administration after 1 h incubation of the cells with H_2O_2) as indicated. 100% represents initial apical gentamicin concentration. Significant differences (*$p < 0.05$) in gentamicin permeation were found between initial value at t=0 h and t=1 h or t= 6 h when insert was not covered with cell monolayer regardless of peroxide administration.

Treatment	Time (h)	Insert without cells		Cell monolayer	
		Apical compartment (%)	Basolateral compartment (%)	Apical compartment (%)	Basolateral compartment (%)
Gentamicin	0	99.85±0.24	0.00±0.29	98.36±0.90	0.15±0.12
	1	72.38±1.07*	26.47±1.52*	99.13±0.85	0.05±0.23
	6	52.13±0.53*	46.81±0.32*	99.94±0.01	1.12±0.81
	0	98.94±0.29	1.02±0.45	99.77±0.96	0.39±0.25
1. 0.5 mM H_2O_2 **2. Gentamicin**					
	1	75.24±2.05*	25.73±1.08*	99,90±0.18	0,19±0.06
	6	54.24±1.83*	45.12±1.25*	99,56±0.83	0,68±0.13
2 phases: **1. 1 mM H_2O_2** **2. Gentamicin**	0	99.91±0.15	0.05±0.03	99,80±0.42	0,23±0.03
	1	73.15±2.16*	27.92±1.86*	99,43±0.96	0.59±0.27
	6	51.26±0.48*	49.95±0.84*	97.79±0.31	3.41±0.15
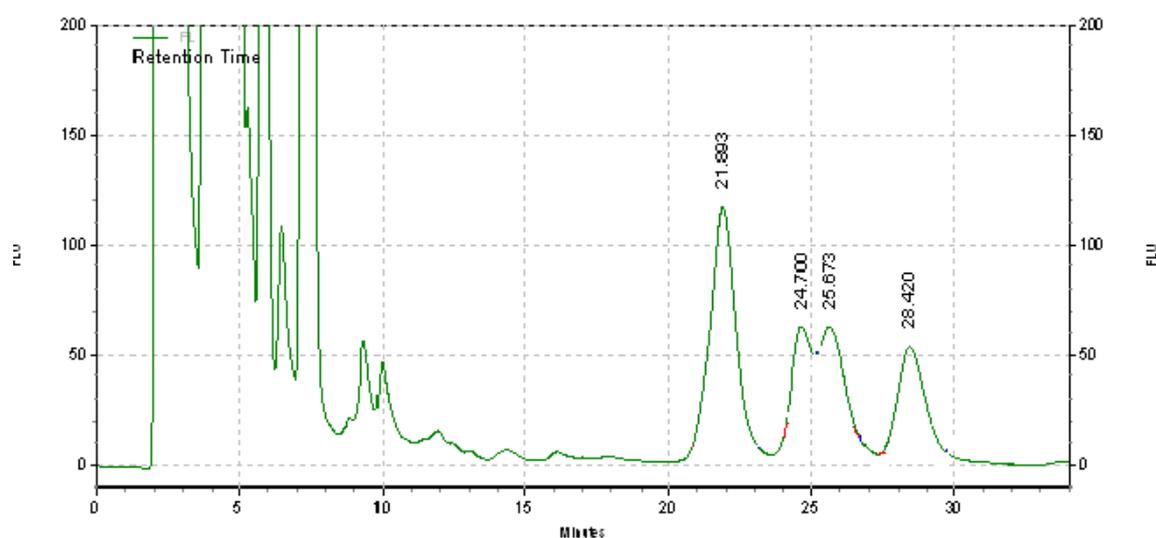
Fig. 22 Representative HPLC elution profile of gentamicin complex collected from apical compartment of IPEC-J2 cell monolayer. The order of elution with mobile phase (85% ACN, 15% UPW) was gentamicin C_1 (t_{ret}=21.89 min), gentamicin C_{1a} (t_{ret}=24.7 min), gentamicin C_{2a} (t_{ret}=25.67 min) and gentamicin C_2 (t_{ret}=28.42 min). The fluorescence detection wavelengths were 260 nm (excitation λ) and 315 nm (emission λ). The flow rate was adjusted to 1ml/min.

3.9. ROS-induced cell death and cytoprotection in undifferentiated IPEC-J2 cells

The dose dependence of H_2O_2-induced cell death in IPEC-J2 cells was examined in 1 h incubations in the presence and in the absence of Ca^{2+} ions by neutral red uptake assay. It was ascertained that 1 h administration of 0.5 mM H_2O_2 in Ca^{2+}-containing medium lead to 64.47±7.78% cell survival, however, in Ca^{2+}-free PBS as low as 0.25 mM H_2O_2 decreased cell viability to 55.74±17.21%. It was also observed that there was no significant change in cell death rates up to 100 μM H_2O_2 for 1 h when Ca^{2+} supplementation at 1.05 mM was maintained in the medium (**Fig. 23**).
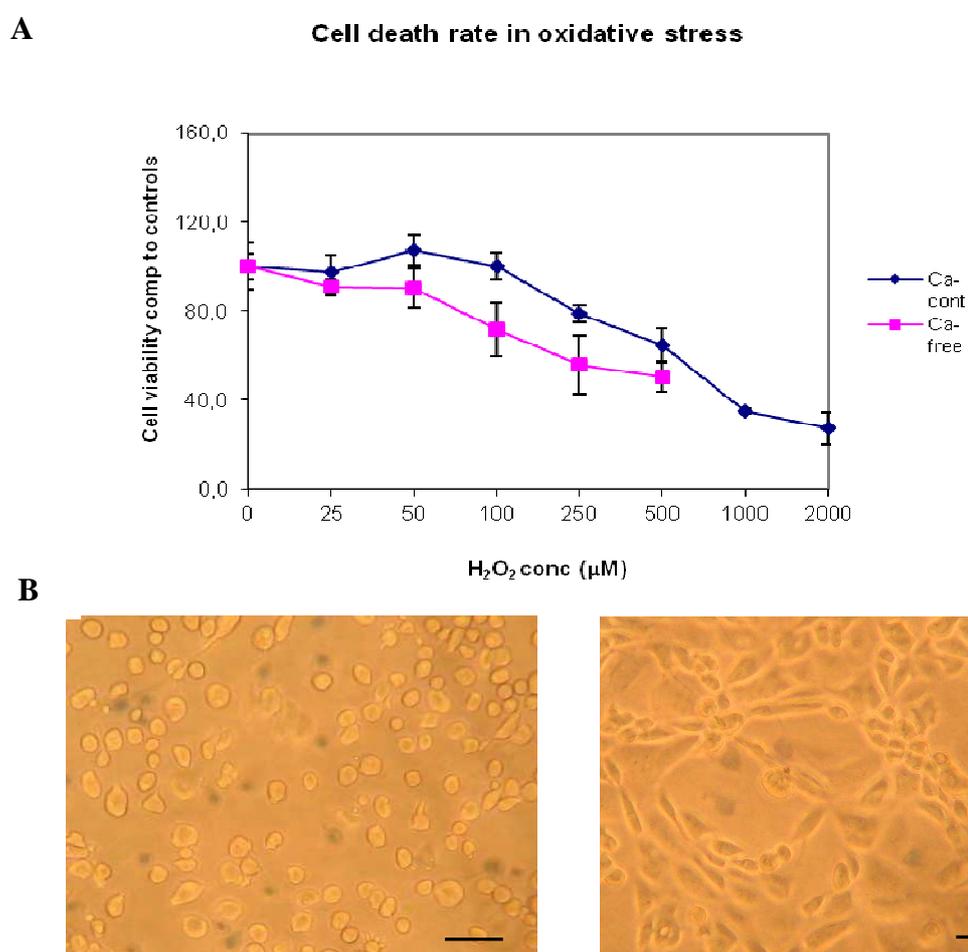
Fig. 23 A H_2O_2 concentration dependence of IPEC-J2 monolayers. Cell viability rate compared to controls *versus* peroxide concentration between 0-2 mM in the absence (pink line) and in the presence of 1.05 mM Ca^{2+} ions (blue line) as determined with the neutral red uptake assay after 1 h incubation. **B** Rounding of the IPEC-J2 cells in Ca^{2+}-free medium (left side) and higher degree of cell adhesion in the presence of Ca^{2+} (right side). Dead cells were stained with trypan blue. Bar indicates 10 μm.

Two independent staining methods suitable for cell viability measurements were applied to estimate the degree of cytotoxicity under basal conditions or when undifferentiated IPEC-J2 cells were exposed to 0.5 mM H_2O_2 and to reveal the putative cytoprotective effect of sodium butyrate added at 2 mM for 1 h. Sodium butyrate could significantly prevent IPEC-J2 cells from oxidative injury and cell death when it was applied simultaneously with 0.5 mM peroxide. Sodium butyrate, when applied alone, did not affect survival of IPEC-J2 cells (**Fig. 24**).
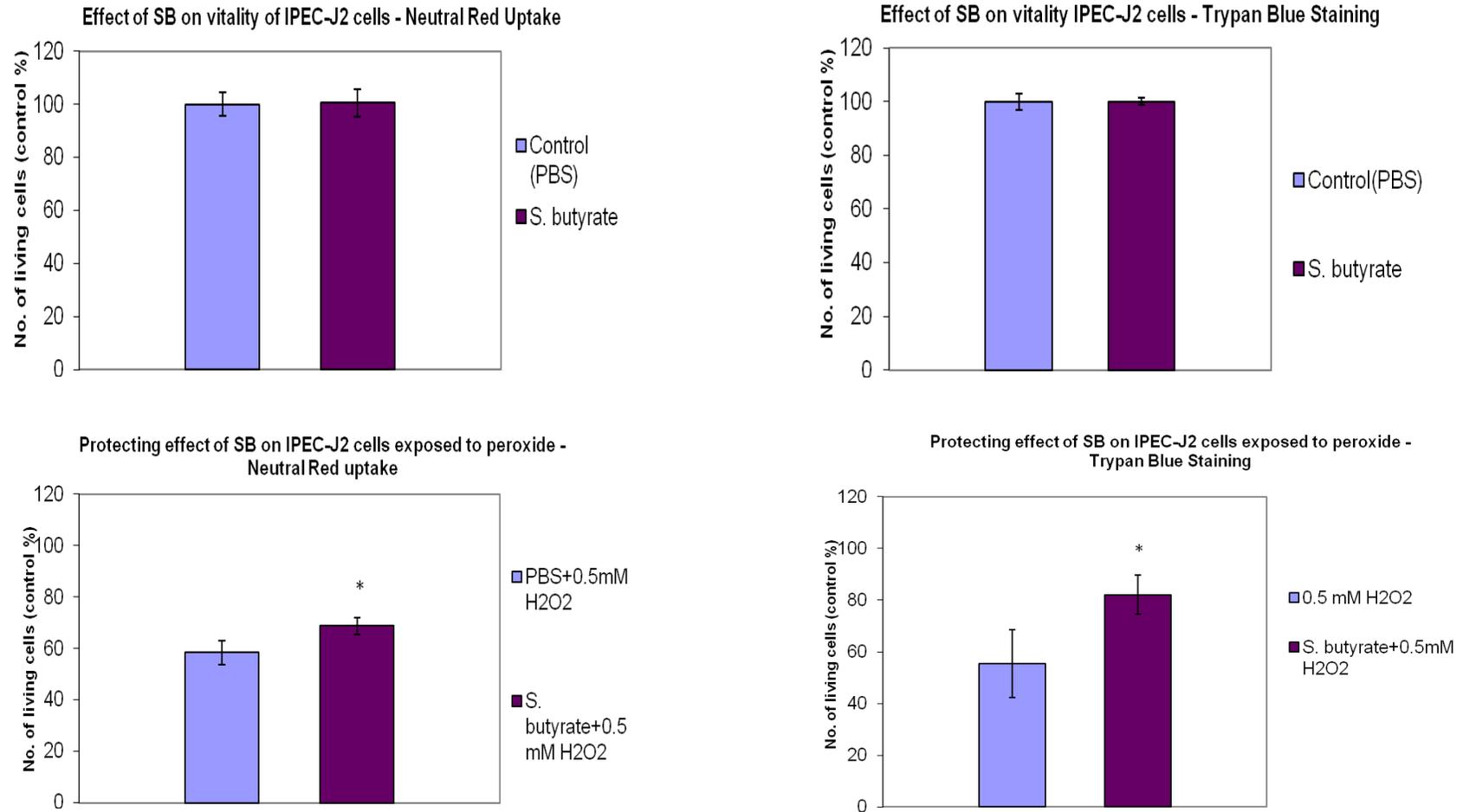
Fig. 24 No change of cell viability after 1 h incubation of undifferentiated IPEC-J2 cells with 2 mM SB was observed. 0.5 mM H_2O_2 treatment-induced cell death was significantly attenuated in undifferentiated IPEC-J2 cells stained with neutral red (left) and trypan blue (right). SB could only prevent oxidative stress induced cell death significantly (* $p < 0.05$).

3.10. Integrity of junctional complexes after ROS treatment

Claudin-1 was detected as a multifocal (non-diffuse) membrane-labelling in IPEC-J2 sample basolaterally. This staining positivity was of the same extension and intensity in three parallel samples. Claudin-4 expression showed diffuse, homogeneous, intense membrane labelling (*data not shown*). Intense membranous positivity of E-cadherin was found in IPEC-J2 cells. Based on the claudin-1 and E-cadherin staining patterns both the expression and the distribution of the investigated proteins were unaltered in the concentration range of 0.5-4 mM H_2O_2 treatments (**Fig. 25**).
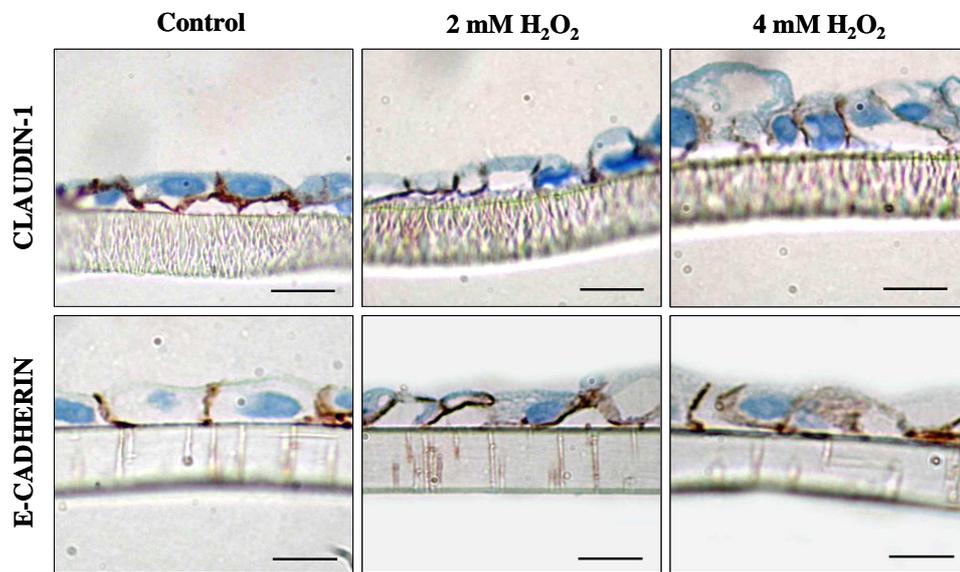
Fig. 25 Immunohistochemistry of claudin-1 and E-cadherin in control and H_2O_2-treated IPEC-J2 cells grown on collagen-coated Transwell polyester membrane insert. Cell monolayers were incubated with 2 and 4 mM H_2O_2 for 1 h. Cells were fixed in 4% buffered formaldehyde solution and labelled for claudin-1 and E-cadherin. IPEC-J2 cells showed homogenous, intense membranous claudin-1 and E-cadherin positivity (brown discoloration). Bar indicates 10 μm.

In semiquantitative immunohistochemical study, claudin-1 negativity was observed in small intestinal samples from ***newborn*** swines (**Fig. 26A**). The enterocytes and stem cells in the crypts showed a diffuse intense lateral membranous linear positivity for claudin-4 (**Fig. 26B**), and the enterocytes on the surface of the villi showed a diffuse punctate (non-linear) membranous positivity for this claudin (**Fig 26C**). The enterocytes on the surface of the villi and the crypt cells showed diffuse intense lateral membranous linear claudin-7 positivity (**Fig. 26D**). In all samples the lymphocytes of the follicles were negative for these claudins, but

false positivity was observed in the protein rich tubulo-vesicular system, lymph, and surface mucin layer. Intense positivity for E-cadherin was detected in enterocytes from jejunum of the carcases of 1-day old newborn unsuckled piglet in accordance with the findings in IPEC-J2 cells.

Claudin-1 negativity was observed in small intestinal samples from *adult* swines (**Fig. 27A**). The enterocytes and stem cells in the crypts showed a diffuse intense lateral membranous linear positivity for claudin-4 (**Fig. 27B**). The enterocytes on the surface of the villi showed diffuse intense lateral membranous linear claudin-7 positivity (**Fig. 27C**) and the crypt cells showed diffuse weaker lateral membranous linear claudin-7 positivity (**Fig. 27D**).

Fig. 26
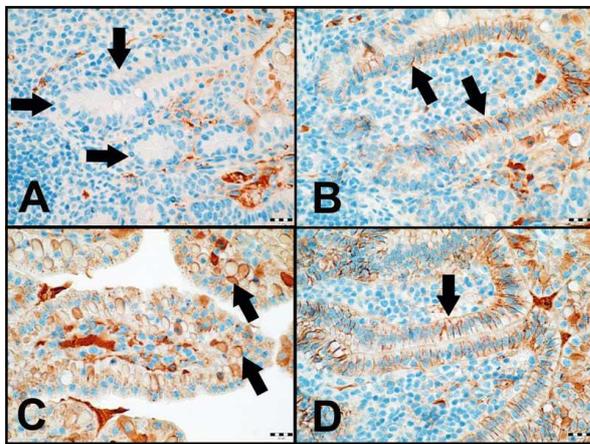
Fig. 27
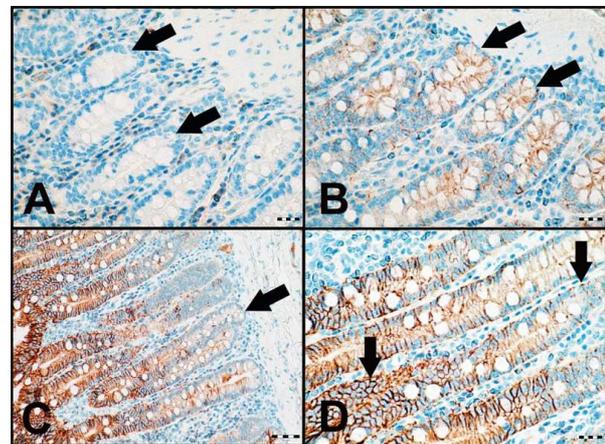
Fig. 26A Claudin-1 negativity in the jejunum of newborn swine (arrows) (IH, 400x, Bar = 20 μm). **26B** Claudin-4 membranous linear positivity in the jejunum of newborn swine (arrows) (IH, 400x, Bar = 20 μm). **26C** False claudin-4 positivity in the tubulo-vesicular system of the apical region of the villi (arrows) (IH, 400x, Bar = 20 μm). **26D** Intense diffuse linear membranous claudin-7 positivity in the buffered formaldehyde-solution fixed and paraffin-embedded jejunal sample (arrows) (IH, 400x, Bar = 20 μm).
Fig. 27A Claudin-1 negativity in the jejunum of adult swine (arrows) (IH, 400x, Bar = 20 μm). **27B** Claudin-4 mebranous linear positivity in the jejunum of adult swine (arrows) (IH, 400x, Bar = 20 μm). **27C** Weak claudin-7 positivity in the crypt cells (arrow) and more intense claudin-7 positivity in the proximal enterocytes of the villi in the jejunum of adult swine (IH, 200x, Bar = 50 μm). **27D** Diffuse linear mebranous claudin-7 positivity in the jejunum of adult swine (arrows) (IH, 400x, Bar = 20 μm).

3.11. Protective effect of sodium butyrate in vitro

Administration of H_2O_2 resulted in decreased TER of IPEC-J2 monolayers grown on Transwell polyester filters coated with rat tail collagen, which could be prevented by simultaneous application of 2 mM sodium-n-butyrate. The protective effect of butyrate was

significant after 24 h recovery (p<0.05), which proved that the monolayer integrity of enterocytes can be maintanined in the presence of butyrate under acute oxidative stress (**Fig. 28**).
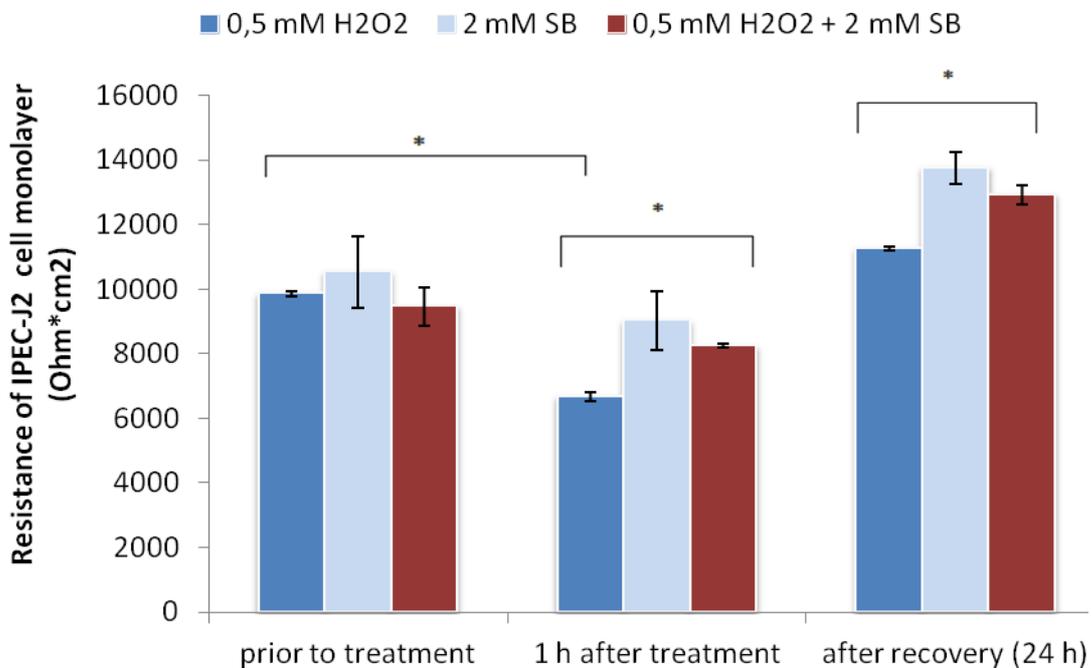
Fig. 28 TER values measured between apical and basolateral compartments of IPEC-J2 cells were determined after treatment with 0.5 mM H_2O_2 alone or together with 2 mM butyrate bolus. The protective effect of butyrate was significant after 24 h incubation time (*p< 0.05). The data were indicated as means ±SEM (n=3).

The growth of *Lactobacillus plantarum* 2142 was indirectly stimulated by sodium-n-butyrate (at concentrations higher than 10 mM) via inhibition of *E. coli* 30037 growth. Significant decrease in the optical density correlated with the number of *E. coli* was found as early as 4 h after 11 mM butyrate treatment. The growth of *Lactobacillus plantarum* 2142 strain was not inhibited up to 100 mM sodium-n-butyrate concentration (**Fig. 29**).

L. plantarum 2142
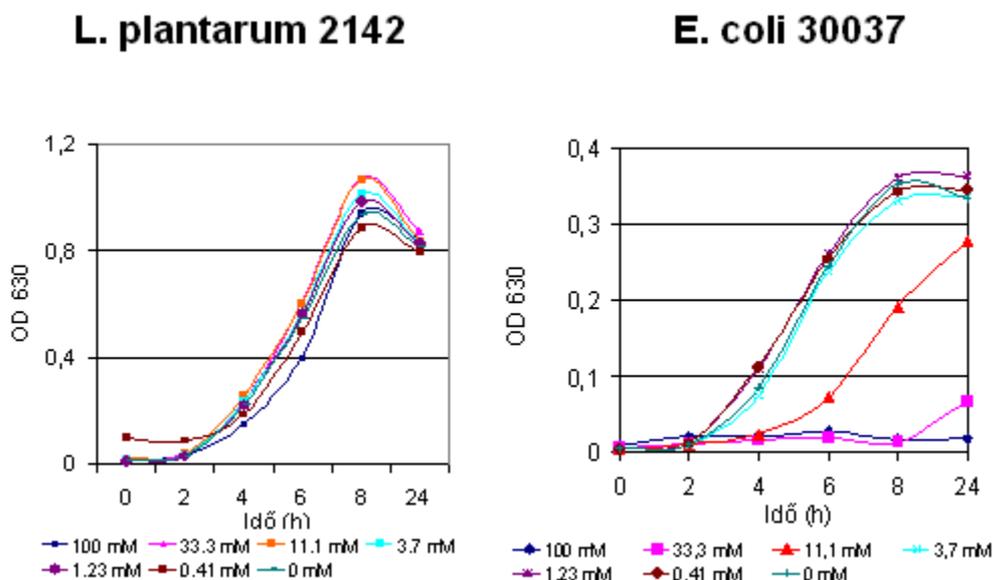
E. coli 30037

Fig. 29 Proliferation of *Lactobacillus plantarum* 2142 (on left side) and *E. coli* 30037 (on right side) under butyrate treatment (butyrate concentration 0-100 mM). Growth was determined by measurement of optical density at 630 nm.

3.12. Butyrate effect in swine ileum

Feed-supplementation of adult swine with 0.15% butyrate for 28 days resulted in an increase in the presence of lactobacilli and secretion of L-lactic acid with concomitant pH decrease in the small intestine. (**Table 9**).

Table 9 The effect of supplemented butyrate (at the concentration of 0.15%) on lactobacilli amount, content of lactic acid and pH in swine ileum

Parameters	Control	Butyrate-treated
Lactobacillus count Log_{10} CFU/g ileal content	8.43 ± 0.32^a	9.29 ± 0.40^b
D-lactic acid $(mM/kg)^2$	1.90 ± 1.80	2.37 ± 2.03
L-lactic acid $(mM/kg)^2$	21.32 ± 3.81^a	30.78 ± 2.12^b
pH^3	6.26 ± 0.16	6.21 ± 0.13

Mean±SEM
[2]mM/kg wet weight
[3]pH of ileal content
[ab]Different letters in the same row indicate significant differences ($p < 0.05$).

It was previously reported that butyrate supplementation did not only increase the count of *lactobacilli* in swine ileum, but also it significantly reduced the number of coliform bacteria (Bokori and Galfi 1990, Bokori et al 1989, Galfi and Bokori 1990). Expression of

Ki67 is characteristic of dividing cells at late G1, at S, G2 and M but not in G0. Based on analysis of 10 microscopic fields the proportion of Ki67 positive cells was determined in crypts. Butyrate supplementation elevated the extent of enterocyte proliferation. The number of microvilli on ileal mucosa was significantly higher when pigs were fed with diet containing 0.15% sodium butyrate. In butyrate-treated animals the number of microvilli was 26.77 ± 2.45 per plica versus 19.30 ± 1.73 in controls (p <0.05). Furthermore, the proportion of Ki67 positive cells was significantly higher (19.6 ± 1.4) in swine with butyrate containing diet compared to control animals (15.7 ± 1.2) (p <0.05) (**Fig. 30**).

| Control | Under butyrate containing diet |
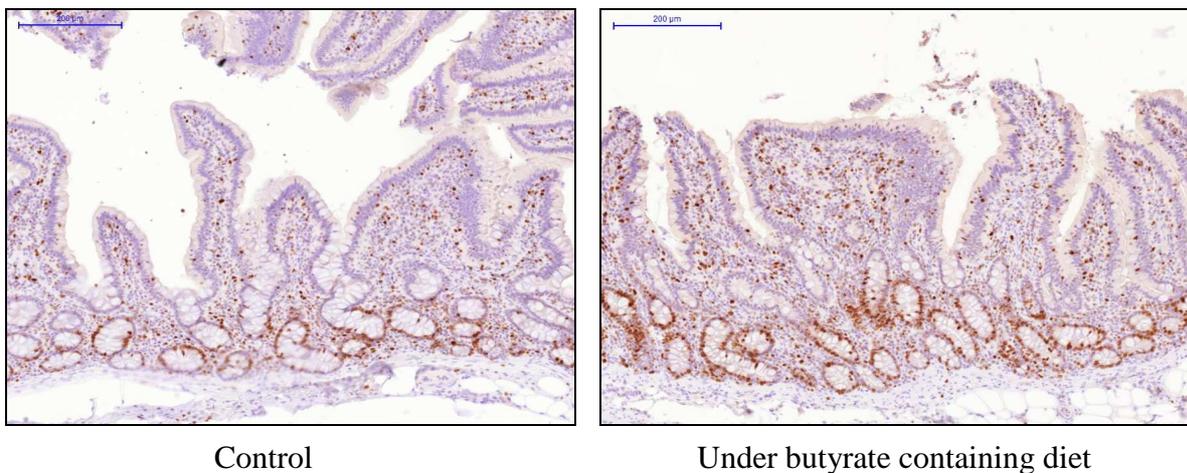
Fig. 30 Increased Ki67 expression in ileum crypt cells of animals under butyrate-diet demonstrates the growth stimulating effect of butyrate. Brown staining indicates Ki67 expressing cells. Bar indicates 200 µm.

3.13. Pattern of PKC isoenzymes in IPEC-J2 porcine epithelial cells exposed to oxidative stress

The PKC α, δ, ε, ζ and η isoenzymes could be detected in IPEC-J2 cells. The lack of PKCγ expression was also confirmed. When cells were exposed to oxidative stress, change in expression of PKC ζ was observed, while no change in the expression level of PKCα, PKCδ and PKCη could be detected (**Fig. 31**).
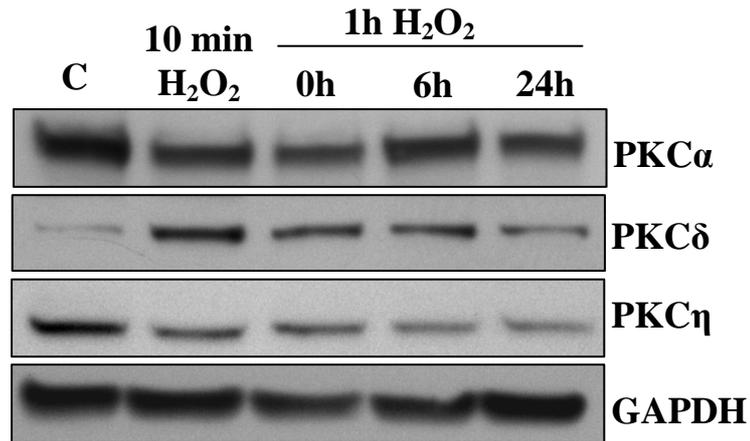
Fig. 31 IPEC-J2 cells cultured on cc-PE for 16-19 days were treated with 1 mM H_2O_2 for 10 min or for 1 h. Expression of PKC isoenzymes (α, δ, η) was examined by Western blot analysis after the 10 min treatment and 0 h, 6 h and 24 h after the 1 h treatment with H_2O_2. GAPDH was probed as a loading control. Representative blots are shown.

Furthermore in differentiated IPEC-J2 cells treated for 1 h with 1 mM peroxide, level of PKCζ increased significantly 6-24 h after peroxide administration i. e. in the recovery period from oxidative stress. (**Fig. 32**).

1h H₂O₂
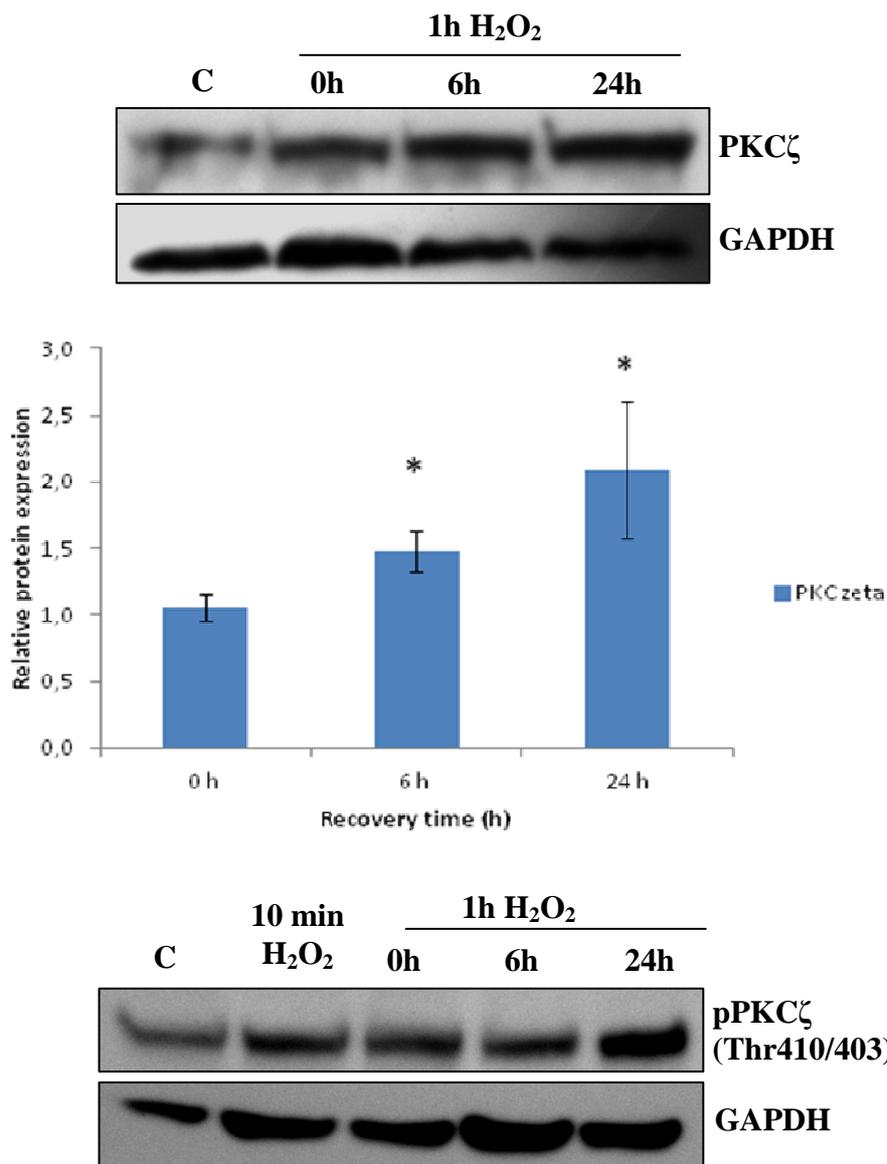
Fig. 32 Phosphorylated forms of PKCζ (upper) and pPKCζ (Thr 410/403) (lower) were examined in IPEC-J2 cells after 10 min or 1 h of 1 mM H₂O₂ treatment with following 6 h and 24 h of recovery time. Threonine phosphorylation was only slightly elevated at 410/403 in PKCζ in IPEC-J2 cells exposed to oxidative stress. GAPDH was probed as a loading control. Representative blots are shown.

3.14. Interaction between SCS of probiotics and hydrogen peroxide

Quantitative determination of hydrogen peroxide using o-DA-based colorimetric method enabled the investigation of putative antioxidant properties of probiotic SCSs. As summarized in **Fig. 33** and **Fig. 34** there was no chemical interaction between H_2O_2 and SCSs of *L. plantarum* 2142, *L. casei* Shirota, *E. faecium* as the peroxide content was not significantly different in SCS-treated and the untreated MRS broth containing control

solutions (P> 0.05) after a 60 min incubation. This clearly indicates that the mode of protective probiotic action can not be explained by antioxidants produced by *L. plantarum* 2142 reducing H_2O_2 chemically in the environment of IPEC-J2 cells changelled with millimolar peroxide, and its anti-inflammatory properties of this probiotic cannot simply be attributed to metabolite-triggered chemical decomposition of the oxidative stress source, H_2O_2.
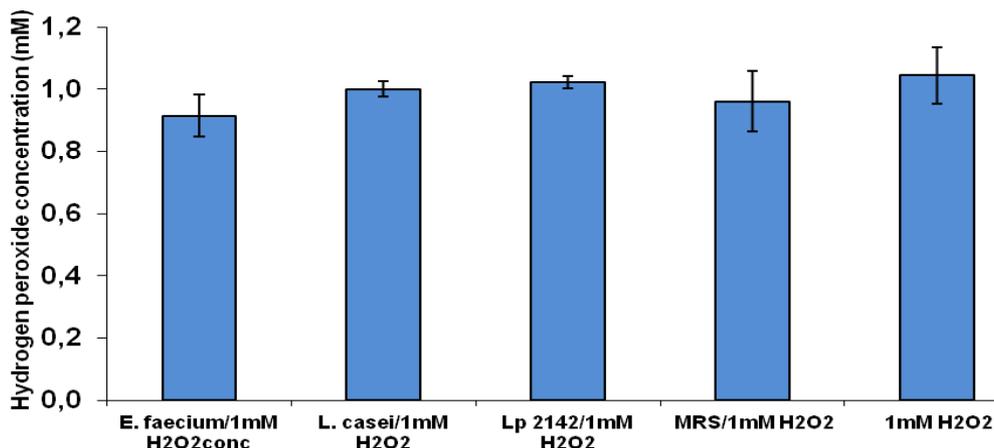
Fig. 33 Effect of SCSs of 5 potential probiotic strains on H_2O_2 levels was determined immediately after treatment using o-DA based spectrophotometric method. There was no significant difference (p> 0.05) between SCSs of *L. plantarum* 2142, *E. faecium*, *L. casei* Shirota and the H_2O_2 control solution.
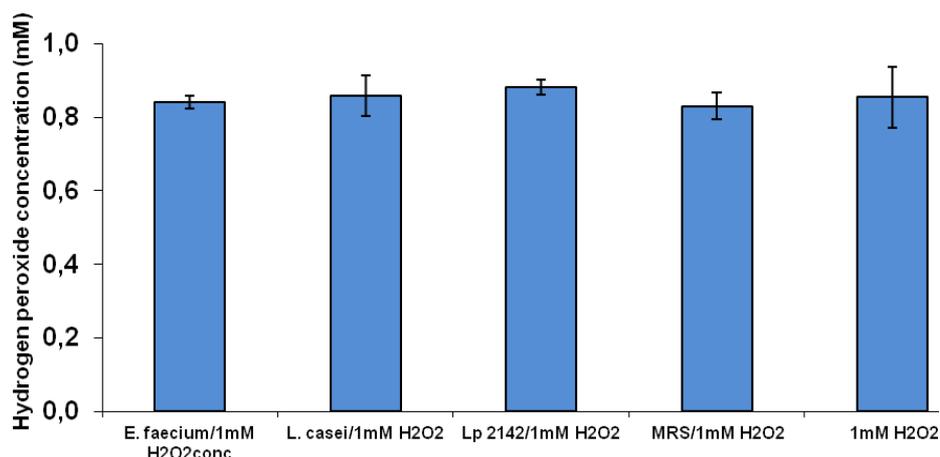
Fig. 34 Supernatants of investigated *lactobacilli* did not cause significant decomposition of the peroxide amount in the solution allowing 1 h reaction time (p>0.05). The growth medium, MRS did also not possess antioxidant properties.

No significant differences in peroxide amount can be detected between solutions containing TPY and *Bifidobacterium animalis* subsp. *lactis* BB-12 SCS immediately and after 1 h, however, using 1 h reaction time the peroxide content was reduced by 37-43% compared to 17% decrease of positive control due to spontanous decomposition of peroxide. The reason of loss in peroxide amount could be the presence of cysteine in TPY which acted as main antioxidant responsible for chemical degradation of aqueous peroxide (*data not shown*). In presence of *B. amyloliquefaciens* SCS almost complete decomposition (above 90% decrease) of peroxide was detected within 60 min reaction time (**Table 10**).

Table 10 *B. amyloliquefaciens* SCS induced almost complete peroxide decomposition in the reaction mixture. TPY, the growth medium of *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) by itself could provoke significant degradation of H_2O_2 due to its cysteine content. TSB was used as growth medium for *B. amyloliquefaciens.** Indicates significant difference between only peroxide-containing positive controls and the screened SCSs or their growth media (p< 0.05).

Composition of solutions for interaction examination	H_2O_2 concentration (mM)		Decrease in H_2O_2 concentration
	Reaction time		Expressed in %
	0 h	**1 h**	
H_2O_2	1.04 ± 0.09	0.86 ± 0.05	17.3
H_2O_2 + TPY	0.86 ± 0.04	0.54 ± 0.07	37.2*
H_2O_2 + SCS (BB-12)	0.96 ± 0.01	0.54 ± 0.11	43.7*
H_2O_2 + TSB	0.72 ± 0.06	0.50 ± 0.05	30.5*
H_2O_2 + SCS (*B. amyloliquefaciens*)	0.48 ± 0.04	0.03 ± 0.001	93.7*

3.15. Screening for the potential anti-inflammatory effect of selected probiotic SCSs (L. casei Shirota, E. faecium, B. amyloliquefaciens and Bifidobacterium animalis subs. lactis BB-12 SCSs) by determining relative mRNA gene expression levels of IL-8 and TNF-α

SCSs of *Enterococcus faecium* CECT 4515 (**Fig. 35A)** and *Bifidobacterium animalis* subs. *lactis* BB-12 (**Fig. 35B**) did not show any attenuating effect on peroxide-induced inflammation since the upregulated TNF-α level did not drop to the pre-inflammation gene expression value. TPY, the growth medium of *Bifidobacterium animalis* subsp. *lactis* BB-12 , alone acted as inflammatory signal as it increased basal TNF-α gene expression. In accordance with the immunmodulatory properties of TPY, 13.3v/v% *Bifidobacterium animalis* subsp. *lactis* BB-12 SCS also stimulated TNF-α expression in peroxide-treated IPEC-J2 cells almost to the same extent.

Peroxide-triggered inflammation could not be quenched via supplementation of *L. casei* Shirota SCS simultaneously added with the 1mM H_2O_2 solution. In spite of decreasing tendency in cytokine expression due to SCS bioactive matrix, no significant difference was detected in relative gene expression of TNF-α between IPEC-J2 monolayers treated with peroxide alone and those treated with peroxide and SCS (**Fig. 35C**).

In contrast, treatment of IPEC-J2 cells with SCS of *Bacillus amyloliquefaciens* CECT 5940 could modulate inflammation induced by 1 mM H_2O_2 administration (**Fig. 35D**). The immunostimulatory effect of the supernatant was manifested in highly elevated IL-8 and TNF- α levels compared when cells were treated with peroxide alone. The highly stimulated gene expression level of these proinflammatory cytokines cannot be attributed to TSB medium, since TNF- α gene expression was increased to the same level by peroxide alone as by peroxide together with TSB. 1 h combined application of 13.3v/v% *B. amyloliquefaciens* SCS containing bacterial metabolites and 1 mM H_2O_2 on IPEC-J2 cells led to 5.7-fold increase in IL-8 and approximately 4-fold increase in TNF-α relative gene expression compared to only peroxide-treated positive controls. Parallel upregulation of these proinflammatory cytokines in IPEC-J2 supports the immunostimulant function of compounds in SCS secreted by *B. amyloliquefaciens* under acute oxidative stress.

35A
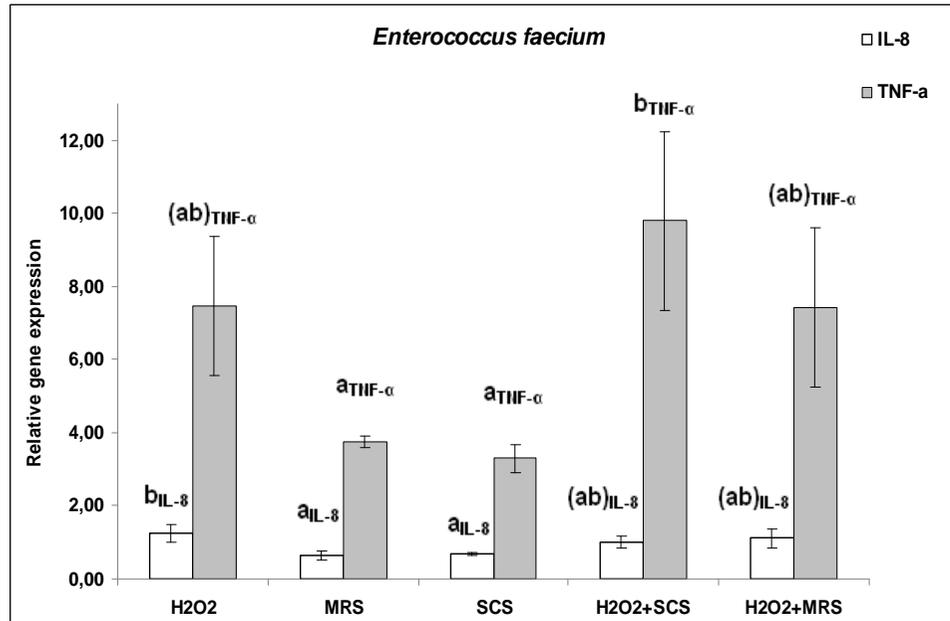
35B
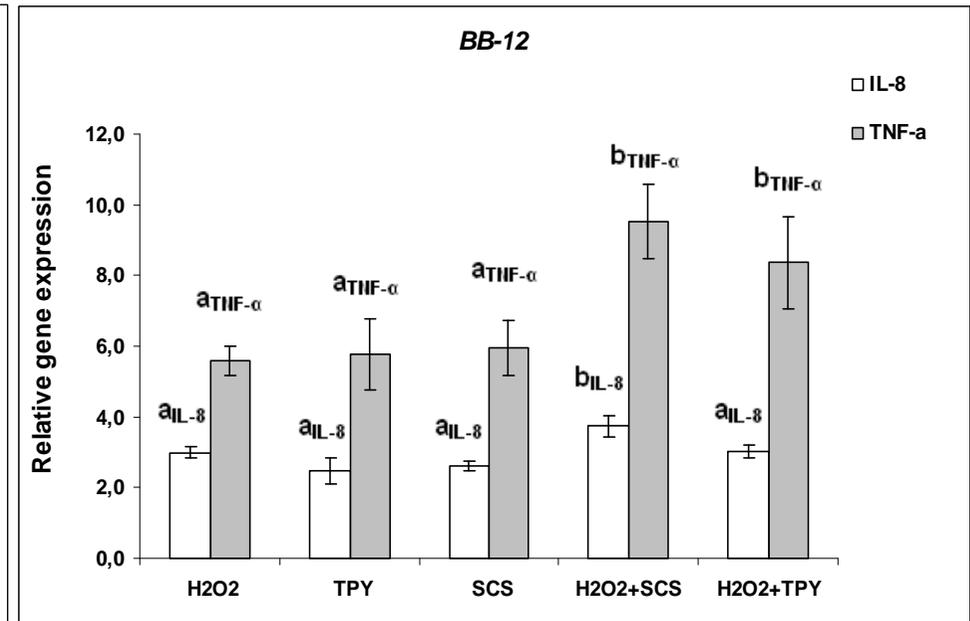
Fig. 35A Peroxide-induced upregulation of proinflammatory cytokine level in IPEC-J2 cells was not influenced by 13.3v/v% SCS of *E. faecium* in plain DMEM applied for 1 h. No significant reduction in IL-8 and TNF-α levels (p> 0.05) was observed by the SCS in the presence of 1 mM H_2O_2

35B Relative IL-8 and TNF-α levels in IPEC-J2 cells after 1 h incubation with SCS from *Bifidobacterium animalis* subsp. *lactis* BB-12 simultaneously with 1mM H_2O_2. No significant difference was found in cytokine relative gene expressions in IPEC-J2 cells exposed to the combinations of 1mM H_2O_2 with TPY or SCS from *Bifidobacterium animalis* subsp. *lactis* BB-12. TPY, growth medium of *Bifidobacterium animalis* subsp. *lactis* BB-12 was used as blank solution in plain DMEM in the same proportion as *Bifidobacterium animalis* subsp. *lactis* BB-12SCS (13.3v/v%).

Data are shown as relative cytokine mRNA level mean± S. E. M. Different letters (a, b, c) show significant differences (p<0.05) (n=6).
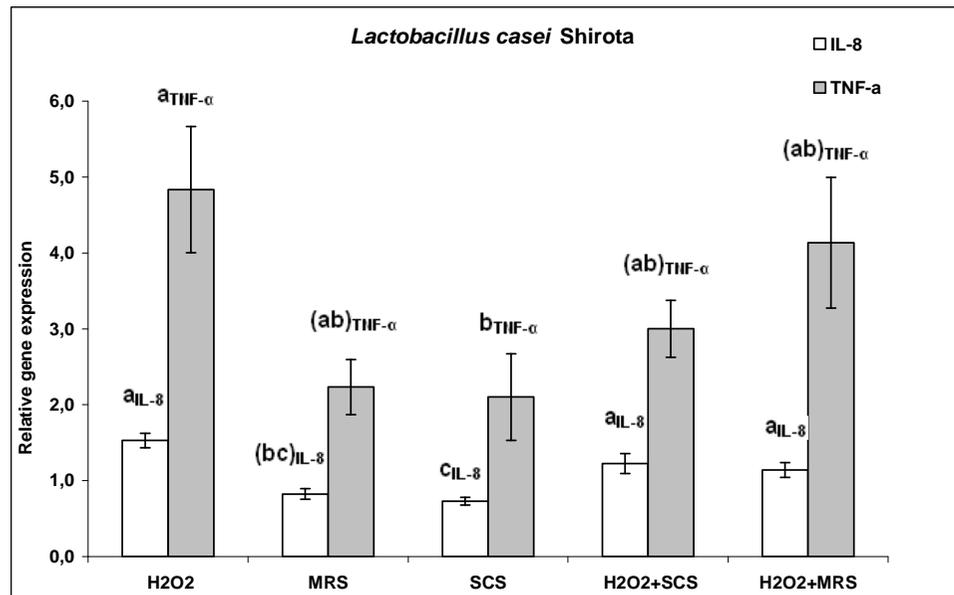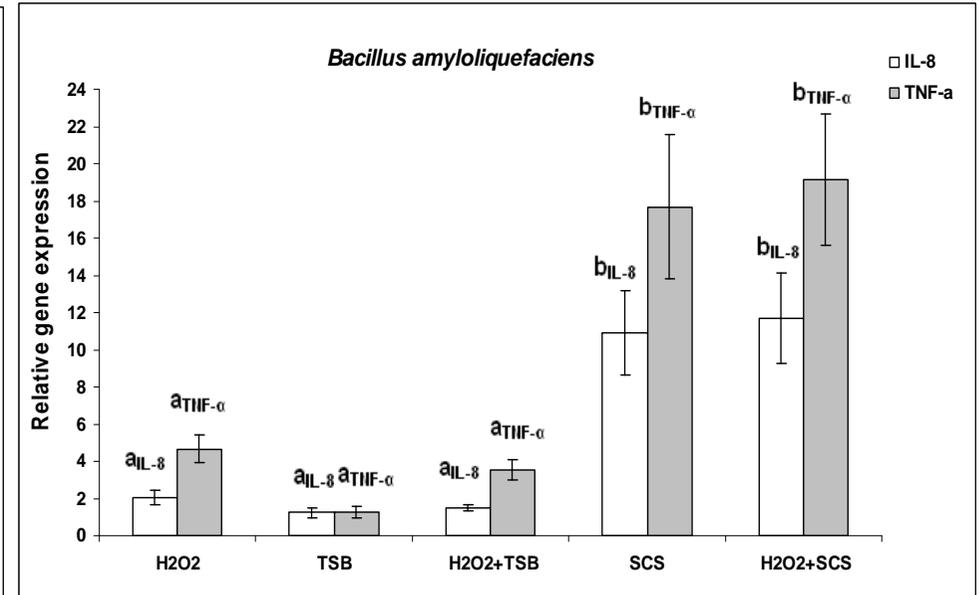
35C Decrease in IL-8 and TNF-α relative gene expression level when 13.3v/v% *L. casei* Shirota SCS in peroxide-triggered acute oxidative stress
35D Potentiation of peroxide-induced IL-8 and TNF-α upregulation was observed when IPEC-J2 cells were incubated for 1 h with SCS of *Bacillus amyloliquefaciens* in 13.3v/v%. Drastic increase in both the IL-8 and TNF- α level was found using qRT PCR technique. Immunostimulant effect of this bacterial SCS is demonstrated.
Data are shown as relative cytokine mRNA level mean± S. E. M. Different letters (a, b, c) show significant differences (p<0.05) (n=6).

3.16. Dose-response relationship of Lactobacillus plantarum 2142 supernatant

Three different concentrations of *L. plantarum* 2142 were used to monitor the changes in acute oxidative stress-induced relative gene expression levels of the proinflammatory cytokines, IL-8 and TNF-α in IPEC-J2 cells exposed simultaneously to probiotic supernatant and peroxide. This should help to determine at which concentration SCS is most effective in quenching oxidative stress-induced inflammation. It was ascertained that supernatant of *L. plantarum* 2142 in 3.3-13.3v/v% affects the relative gene expression of proinflammatory cytokines such as IL-8 and TNF-α differently in peroxide-treated IPEC-J2 cells. Higher dose of probiotic SCS could suppress the peroxide-triggered upregulation of IL-8 and TNF-α significantly in contrast to supernatants applied in 3.3 and 6.6v/v% (**Table 11**). 13.3 v/v% *L. plantarum* 2142 SCS and its culturing broth, MRS did not influence significantly the basal IL-8 and TNF-α level of IPEC-J2 cells.

Table 11 Relative gene expression of IL-8 and TNF-α in IPEC-J2 cells treated with H_2O_2, *L plantarum* 2142 supernatant and MRS, mean ±SEM, n=4. *indicates significant difference compared to those treated with H_2O_2 alone.

	IL-8	TNF-α
1 mM H_2O_2	2.9± 0.6	6.1 ± 1.0
13.3v/v% MRS	0.9 ± 0.2*	2.4 ± 0.1*
13.3v/v% SCS	0.9 ± 0.2*	2.7 ± 0.9*
1 mM H_2O_2 + 3.3v/v% MRS	3.7 ± 0.5	6.4 ± 0.5
1 mM H_2O_2 + 3.3v/v% SCS	3.1 ± 1.4	5.3 ± 2.0
1 mM H_2O_2 + 6.6v/v% MRS	3.7 ± 0.9	6.9 ± 1.0
1 mM H_2O_2 + 6.6v/v% SCS	2.9 ± 0.5	7.3 ± 2.4
1 mM H_2O_2 + 13.3v/v% MRS	1.9 ± 0.3	6.9 ± 0.4
1 mM H_2O_2 + 13.3v/v% SCS	1.0 ± 0.2*	2.8 ± 0.6*

3.17. Protection against oxidative stress by application of L. plantarum 2142 SCS application

The maximal protection against 1mM H_2O_2–triggered inflammation in IPEC-J2 cells was achieved using 13.3 v/v% *L. plantarum* 2142 (**Fig. 36**) as the gene expression level of TNF-α remained as low as it was characteristic of 13.3v/v% MRS broth and SCS in the absence of peroxide. Peroxide-triggered TNF-α relative gene expression was attenuated by 1 h 13.3v/v% SCS treatment from 7.514±1.483 to 2.902±1.041. At the same time, significant reduction in IL-8 level (from 2.645±0.901 to 1.508±0.534) was also observed by *L. plantarum* 2142 SCS. In contrast, treatment with SCS in lower doses (3.3 and 6.6 v/v%) did not result in a significant beneficial effect against acute oxidative stress. TNF-α gene expression upregulated by peroxide did not change significantly when IPEC-J2 cells were challenged with peroxide together with a low-dose *L. plantarum* 2142 supernatant in plain DMEM. TNF-α levels decreased to 7.298± 2.431 when *L. plantarum* 2142 was applied at 3.3 v/v% and it was further attenuated to 6.997 ± 0.419 by a 2-fold concentrated supernatant.
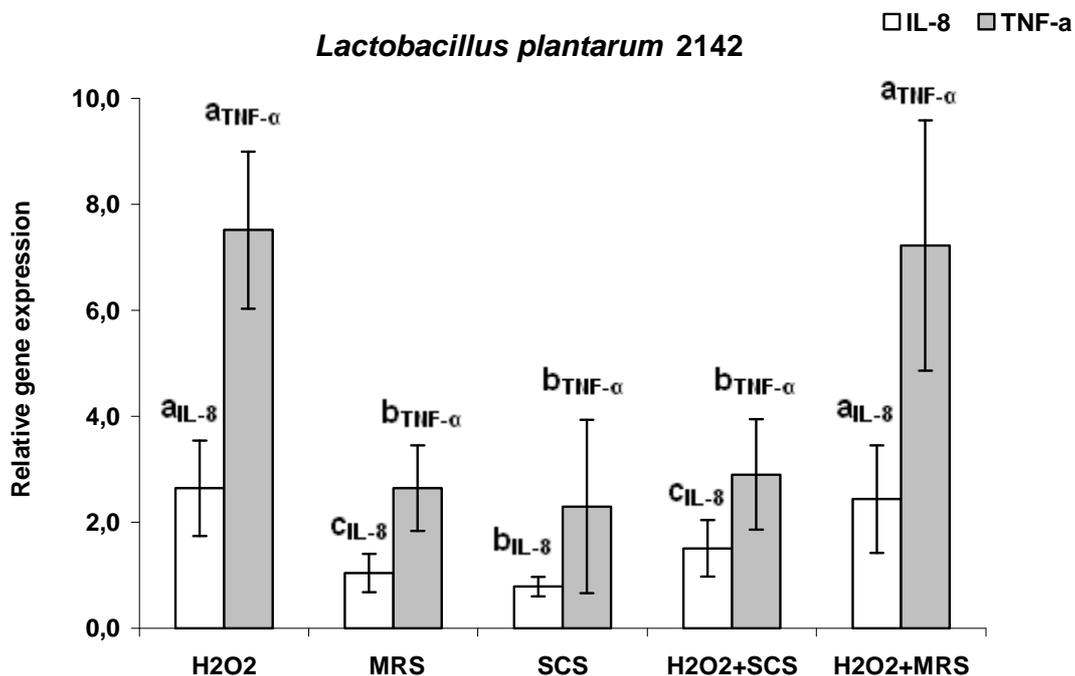
Fig. 36 Protective effect of *L. plantarum* 2142 SCS against 1 mM H_2O_2-induced IL-8 and TNF-α upregulation in IPEC-J2 cells. Significant decrease in level of proinflammatory cytokines occured when SCS was added at 13.3v/v% in plain DMEM (n=6). Different letters visualize pairwise statistical differences obtained from Tukey post-hoc test in each cytokine group (p<0.05). Data are shown as investigated cytokine level means± S. E. M.

By the addition of 1 mM H_2O_2 to the IPEC-J2 cells the basal gene expression of Hsp70 was upregulated by more than 2.5-fold. This elevation was further potentiated when 13.3 v/v% *L. plantarum* 2142 SCS was applied simultaneously with peroxide leading to a higher than 7-fold increase in Hsp70 level compared to control groups (**Fig. 37**). When only SCS was added, slight but statistically significant increase in Hsp70 relative gene expression was detected. MRS, however, counteracted the Hsp70 gene transcription promoting effect of oxidative stimuli partially explained by its impact on the basal expression of Hsp70 mRNA. These results suggest that an anti-inflammatory route, of *L. plantarum* 2142 SCS could involve upregulation of Hsp70 with parallel attenuation of expression of the proinflammatory cytokines.
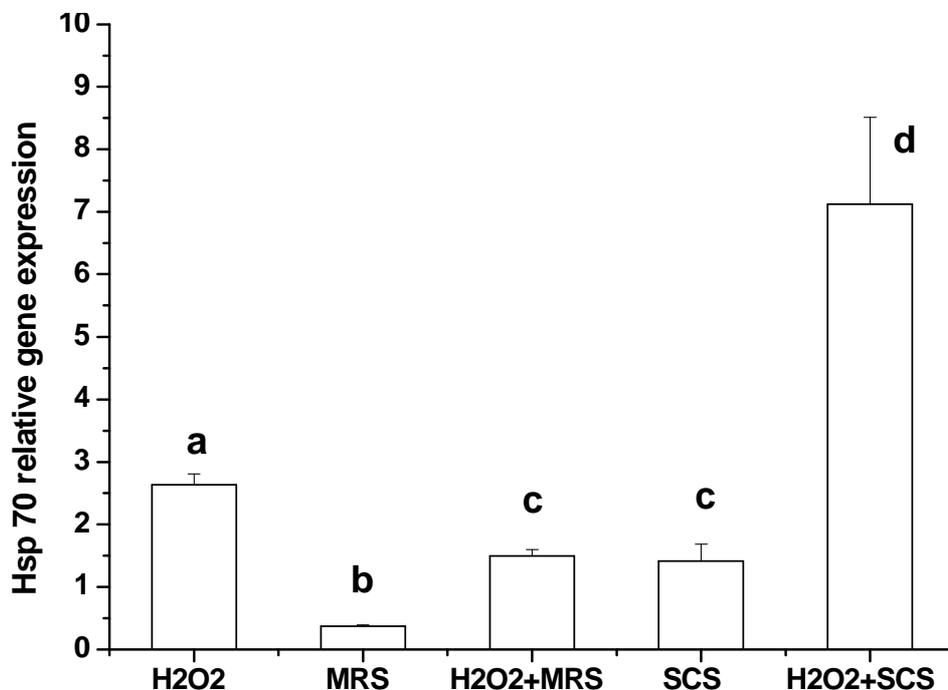
Fig. 37 Changes in Hsp70 relative gene expression levels in IPEC-J2 cells treated with 1mM H_2O_2 alone and in combination with 13.3v/v% MRS or *L. plantarum* 2142 SCS, in the presence of MRS and SCS. Significant differences in Hsp70 levels were observed in three parallel experiments between groups labelled with different letters (p< 0.05).

Polarized IPEC-J2 cells with TER higher than 8000 Ohm*cm^2 were used for every experiment. TER values prior to and post experiment were compared to check if cell integrity was influenced by probiotic treatment during a 60 min incubation time. As shown in **Fig. 38** statistically significant change did not occur in TER after application of *L. plantarum* 2142 SCS even at the highest concentration. This confirmed that 13.3v/v% MRS, Lp 2142 SCS

alone and in combination with 1 mM H_2O_2 did not cause damage to integrity of the IPEC-J2 cell monolayer cultured on mebrane inserts.
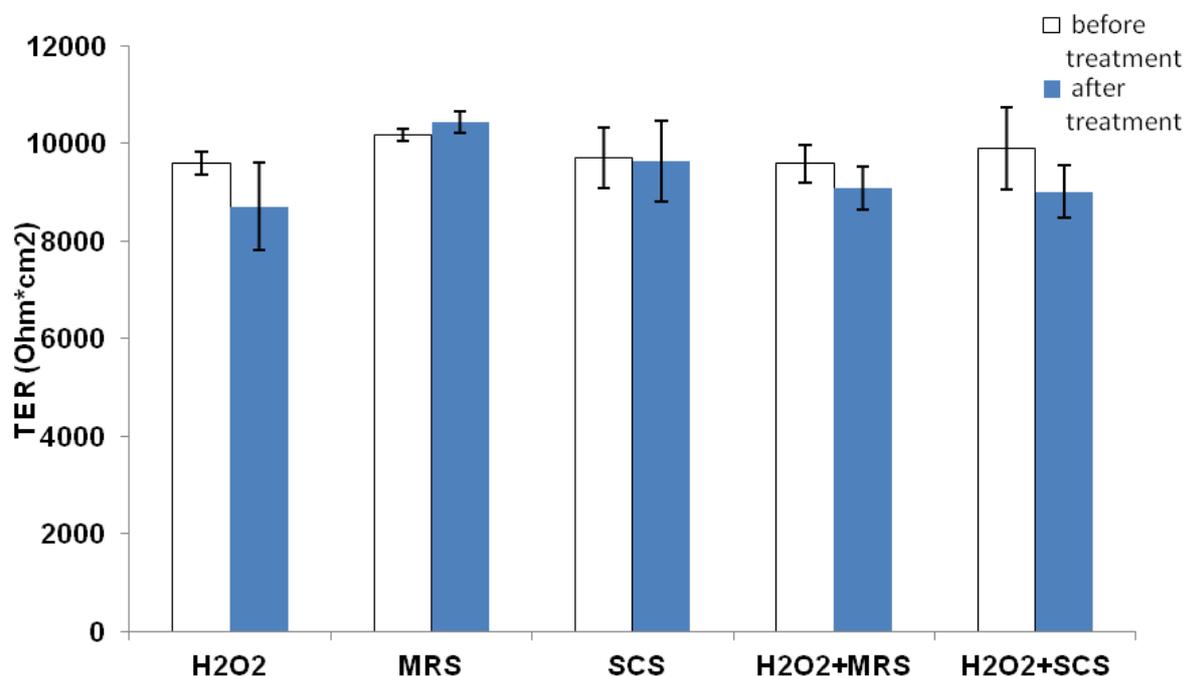
Fig. 38 TER values measured on IPEC-J2 cell monolayers did not change by the administration of MRS, *L. plantarum* 2142 SCS alone or together with peroxide ($p> 0.05$). *L. plantarum* 2142 was cultured in MRS broth. Data are shown as TER mean values ± S. E. M (n=3).

3.18. Role of fatty acids in the anti-inflammatory action of L. plantarum 2142 action

The presence of SCFAs among lactobacilli metabolites caused a dramatic drop in pH value of the probiotic supernatants. The pH value in *L. plantarum* 2142 SCS was remarkably low (pH=4.3) maintaining a strong acidic environment under which IPEC-J2 cells could not survive based on trypan blue exclusion assay (> 95% cell death rate).

When SCS was applied at effective dose against oxidative stimuli it was slightly acidic medium (pH=7.1) and thus could be applied without disturbing the physiological activity of IPEC-J2 cells. The heterofermentative production *L. plantarum* 2142 mainly involves secretion of the two carboxylic acids, lactic acid (LA) and acetic acid. The quantitative analysis of D- and L-LA (D-LA and L-LA) content in lactobacilli SCS (**Fig. 39**) proved that the ratio of 2 enantiomers varies with the type of the bacterial strains selected for the investigation. It was shown that the D-LA production of *E. faecium* is negligible (less than

1mM) compared to the increased D-LA level detected in *L. plantarum* 2142 (97 mM) and *L. casei* Shirota (91 mM). L-LA content is less variable, it ranges between 48-67 mM, the lowest quantity was detected in *E. faecium* SCS.
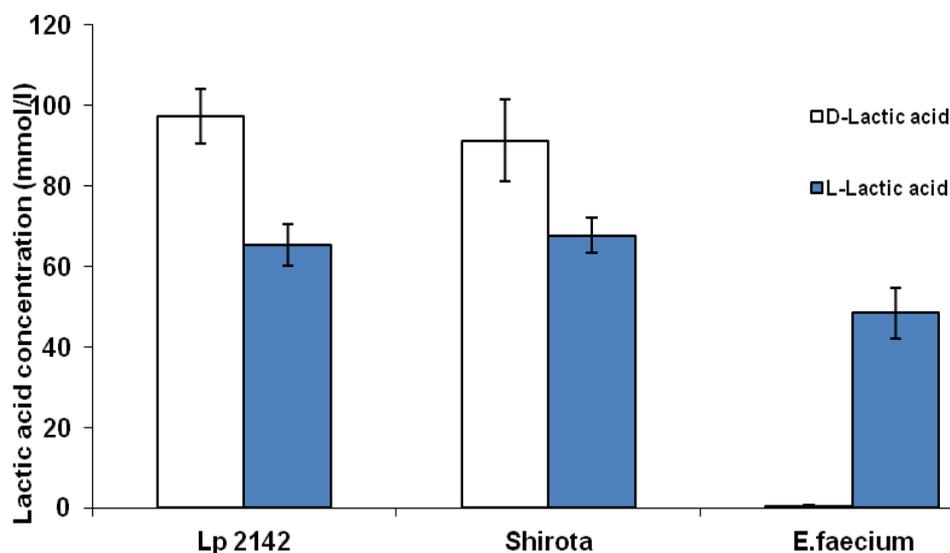
Fig. 39 D- and L-lactic acid concentrations in media of *L. plantarum* 2142 (Lp 2142), *L. casei* Shirota (Shirota) and *E. faecium* SCS were determined with a lactate dehydrogenase-based detection kit (n=3). The mean values ± SEM are expressed in mmol/l.

To identify active components in the *L. plantarum* 2142 supernatant, L-LA (65 mM), D-LA (100 mM) and their 2:3 racemic mixture were added to IPEC-J2 cells in 13.3 v/v% concentration to determine if the short-chain carboxylic acids are partially or exclusively responsible for beneficial anti-inflammatory effect of *L. plantarum* 2142 (**Fig. 40**). Addition of D-LA, L-LA and their racemic mixture in the concentration present in *L. plantarum* 2142 SCS to cell medium did not reduce significantly peroxide-induced increase in IL-8 and TNF-α relative gene expression pointing out that the major downregulators of oxidative stress-induced response are not these organic acids in a probiotic supernatant. Furthermore, Hsp70 level remained unchanged when LA enantiomers of the racemic mixture were added to IPEC-J2 cells post H_2O_2 treatment. Thus, LA had no protective effect against oxidative stress-mediated changes in IL-8, TNF-α and Hsp70 gene expression levels.
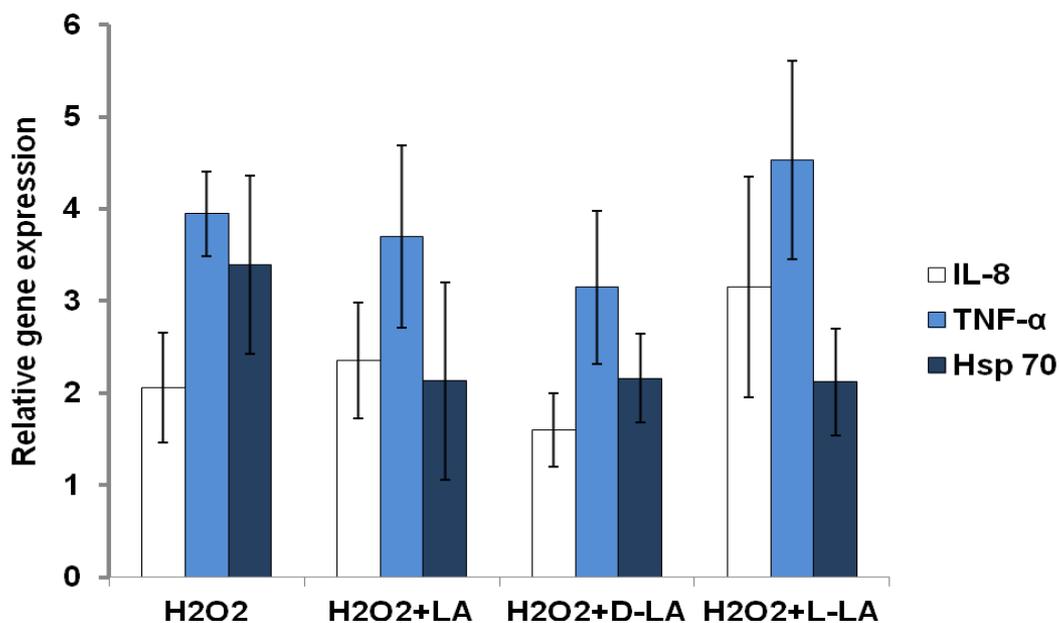
Fig. 40 Effects of racemic, D- or L-lactic acid (LA) on peroxide-induced proinflammatory cytokine upregulation. No significant beneficial effect (p> 0.05) was found such as quenching of peroxide-induced upregulation of IL-8, TNF-α and Hsp70 gene expression level in the presence of lactic acid (n=7).

The another short chain carboxylic acid, acetic acid at 1 mM and 7 mM concentrations (pH=7.3-7.4) did not exert beneficial effect on acute oxidative stress as it did not decrease elevated IL-8 and TNF-α level induced by 1 mM H_2O_2 significantly (**Fig. 41**).
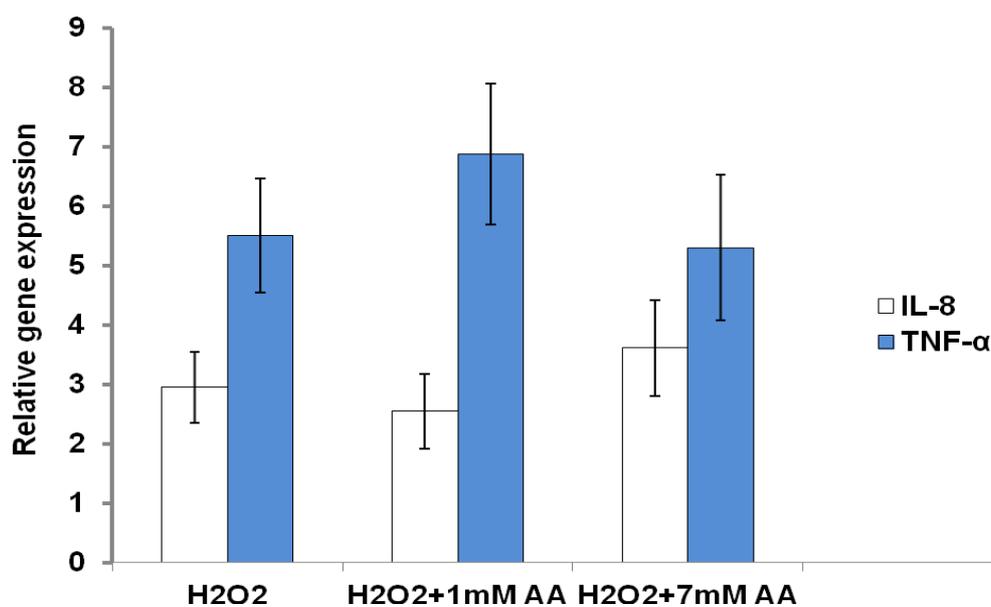
Fig. 41 Acetic acid (AA) at 1 mM and 7 mM did not affect relative gene expression levels of IL-8 and TNF-α upregulated by a 1 mM peroxide treatment for 1 h. The values are given as means ±SEMs (n=3).

4. Discussion

Determining the oxidative stress-induced harmful effects at molecular level and in the search for potential scavenger molecules, probiotic metabolites are promising targets in human as well as veterinary medicine. Probiotics play an important role in control of inflammatory processes and represent a treatment option in degenerative diseases which develop as a long-term consequence of excessive oxidative stimuli. Oxidative stress is caused, on one side, by reactive oxygen intermediates which are generated in tissues during aerobic respiration and inflammation, and on the other, by ROS derived from environmental sources [Schroder and Krutmann 2005, Finkel and Holbrook 2000, Harris et al 1992] such as in the gastrointestinal tract, where different levels of ROS can be produced in gut lumen. Potential sources of luminal oxidants can be xenobiotics, endotoxins, oxidized food debris, and higher levels of iron ions and bile acids [Blau et al 1999, Giandomenico et al 1997].

In our model H_2O_2 was applied to generate oxidative stress. H_2O_2 is often used in studies of redox-regulated processes because it is relatively stable compared to other ROS. In order to estimate the amount of H_2O_2 that was available for the cells, decomposition of H_2O_2 was determined in cell-free plain medium, and also consumption of H_2O_2 was measured in the culture medium in the presence of IPEC-J2 cells. In distilled water, decomposition of H_2O_2 was not significant. In plain medium, however, there was a reduction by 21% of the initial H_2O_2 after 1 h of incubation.

IPEC-J2 cell line derived from jejunal epithelia isolated from a neonatal piglet and human colon adenocarcinoma Caco-2 cell line were used for studying on interaction of oxidative stress and probiotics on normal intestinal epithelium. To mimic *in vivo* conditions they were cultured on Transwell membrane inserts which allowed them to reconstruct the polarized functional epithelia and form TJs *in vitro*. Tumor-derived cell lines are heterogeneous [Sambuy et al 2005], which explains that considerable differences may exist between Caco-2 strains from different laboratories. TER values of Caco-2 cells are highly variable compared to those measured with IPEC-J2 cells when both were cultured on membrane inserts. Additionally, cell passage dependent protein expression or other functional changes were also documented since in case of Caco-2 cells there were inter-laboratory variations in transepithelial resistance as much as 20-fold [Hidalgo 2001].

Viability of the cells is decreased in a ROS concentration-dependent manner due to the cell-damaging properties of the ROS as it was confirmed using different types of molecular stains. DAPI binds to the nuclei of cells only after the membrane becomes

permeable, therefore this method was applied to assay the level of cell death after H_2O_2 treatment. Studying early effects of H_2O_2 on filter-grown Caco-2P cells, it was found that 1 to 4 mM H_2O_2 had no impact on cell viability, while 20% of nuclei were stained with DAPI after 10 mM H_2O_2 exposure. However, IPEC-J2 cells seem to be more sensitive since 10% and 15% of the differentiated IPEC-J2 cells lost their viability after 2 mM and 4 mM H_2O_2 treatment, respectively [Nemeth et al 2007].

Epithelial barrier disruption as a result of damage to junction protein complexes can lead to changes in paracellular permeability of intestinal epithelial cells which can be followed by detecting TER [Rao et al 1997]. Decrease in TER may also reflect an increase in movement of solutes and ions across the epithelium by transcellular pathways. Remarkable difference in reactivity towards oxidative stress could be found comparing these two cell lines; since differentiated IPEC-J2 cell monolayer integrity can be partially disrupted by 1 h 2 mM hydrogen peroxide administration in contrast to filter-grown Caco-2P cells, where 4 mM H_2O_2 treatment did not exert impact on cells, and more than 10 mM H_2O_2 for an h could only decrease TER significantly. Membrane damage observed as a consequence of 2 to 4 mM H_2O_2 treatment in differentiated IPEC-J2 cells suggests that TER decrease can be partly attributed to ion movement via transcellular pathways.

For optimization of IPEC-J2 cell culturing conditions, PC and cc-PE membrane inserts were tested and compared in 3D functional cell model. TER change in the linear growth phase (between the 9th and 19th days of culture) was higher on cc-PE, than those on PC membrane inserts. Furthermore, final TERs (which were not as variable as in case of Caco-2 cells) were higher on cc-PE than on PC inserts. In order to provide higher degree of differentiation of IPEC-J2 cell, cc-PE membrane inserts were applied throughout the study. The resemblance of IPEC-J2 cells to gut makes this 3D model capable of predicting physiological and redox cellular changes in jejunal epithelium exposed to inflammatory processes of oxidative origin [Paszti-Gere et al. 2012].

ROS can react with proteins, lipids and nucleic acids resulting in the development of dysfunctional proteins, membrane lipid peroxidation and DNA fragmentation [Valko et al 2007]. The outcome of the oxidative stress (from alteration of gene expression profile, programmed cell death to passive cell death due to catastrophic injury) greatly depends on ROS concentration and exposure time, cell type, and the actual antioxidant capacity of the cell [Saberi et al 2008]. It was supposed that one of the most characteristic consequences of oxidative stress-triggered damage would be the elevated rate of lipid peroxidation dependently of applied peroxide concentration (0-4 mM) and the gradual decrease in capacity

of cellular antioxidant defence mechanisms (GSH, GPx). However, no significant change occurred in the level of any investigated antioxidant parameters, neither in concentration of early stage lipid peroxidation indicators, such as CDs, CTs nor in amount of late phase marker, MDA by the effect of oxidative intervention.

Pro-inflammatory factors such as enteroinvasive *Escherichia coli*, oxidative stress induced by xanthine oxidase and xanthine or H_2O_2 and pro-inflammatory cytokine TNF-α have been shown to cause rearrangement and decreased expression of TJ and AJ proteins in Caco-2 cells and MKN28 gastric epithelial cells [Seth et al 2008, Miyauchi et al 2009, Qin 2001]. This was also found in case of dextran sodium sulfate-induced colitis in mice [Mennigen 2009]. In the present study IPEC-J2 cells showed homogenous and intense membranous claudin-1 and E-cadherin positivity confirming the development of differentiated phenotype. After a 1 h treatment with H_2O_2, up to the concentration of 4 mM, the cellular distribution and staining expression of these proteins remained unaltered. This seems to suggest that the TER decrease observed in the concentration range 2-4 mM H_2O_2, can either be attributed entirely to cell damage or disruption of junction proteins other than claudin-1, claudin-4 and E-cadherin.

Transcellular permeation of gentamicin is practically not possible in view of its relatively high molecular weight, highly polar and hydrophilic nature [Rama Prasad et al 2003]. We proved that gentamicin also showed poor paracellular permeation across TJs across the IPEC-J2 monolayer. In accordance with these findings gentamicin could be used for detecting potential epithelial barrier dysfunction after acute oxidative stress based on chromatographic separation and quantitative fluorometric determination of gentamicin collected from basolateral compartment of IPEC-J2 cell monolayer exposed to peroxide treatment. We ascertained that 1 h peroxide administration up to 1 mM concentration did not alter the physical barrier function and integrity of cell monolayer since only negligible amount of gentamicin could transport into the basolateral compartment.

The signalling pathways triggered by acute oxidative stress were also investigated with analysis of the expression pattern of PKC isoenzymes. In differentiated IPEC-J2 cells, PKC α, δ, ε, ζ, és η isoenzymes could be detected. PKCγ was not detected in differentiated IPEC-J2 monolayer. When differentiated IPEC-J2 cells were treated with 1 mM peroxide for 1 h, expression level of PKCζ increased significantly 6-24 h after peroxide administration during the recovery period, suggesting that signal transduction pathways behind potential anti-inflammatory effect of protective substances such as probiotics under acute oxidative stress may involve regulatory mechanisms through PKC ζ.

Epithelial cells were found to secrete a great variety of cytokines functioning as growth and differentiation factors, alarm signals for the inflammatory response, chemotactic factors and modulators of immune cell function [Stadnyk 1994]. "Cell stressors" such as heat-shock, hyperosmolarity, adherence of a foreign surface and ROS trigger the gene expression of pro-inflammatory cytokines which initiate the local and systemic immune response in order to defend the host against the noxious stimuli [Dinarello 2000]. It was found [Son et al 2005] that H_2O_2 and TNF-α induced potent neutrophil chemoattractant and activator, IL-8 secretion in Caco-2 and HT-29 cells. After short-term stimulation of oxidative stress induced by 1 mM H_2O_2 IL-8 mRNA expression increased in Caco-2 cells and in normal intestinal epithelial cells, ACBR I519 [Yamamoto et al 2003]. In our work dose-and time-course response of cytokine expression were determined in filter-grown IPEC-J2 cells after H_2O_2 treatment. ROS treatment increased the gene expression level of IL-8 and TNF-α, but left IL-6 expression unchanged. The most effective concentration in stimulating both IL-8 and TNF-α expression was 1 mM H_2O_2. Decrease over the higher concentration range may be due to oxidative damage of cell functions such as transcription and also to oxidative cell death. Similar observations were made in Caco-2 cells and MKN28 gastric epithelial cells, where maximal IL-8 gene expression was also evoked by 1 mM H_2O_2 treatment [Shimada et al 1999, Yamamoto et al 2003]. The short-term time-course assay of IL-8 and TNF-α expression level due to 1 mM H_2O_2 treatment showed that it increased gradually as the cells consumed the H_2O_2. Gene expression levels of IL-8 and TNF-α peaked after 1 h of recovery time, then they started to decrease and reached the control level after 6 h.

In addition to study on relative gene expression of IL-8 by qRT-PCR, secretion rate of IL-8 was also followed by ELISA measurement, which confirmed the release of this cytokine into the apical medium only. IL-8 concentration was significantly elevated at 4 h after peroxide administration and it increased continuously until the end of recovery time. After 24 hs of recovery 101 pg/ml IL-8 was detected in the apical medium of IPEC-J2 cells. Exposure of Caco-2H cells to millimolar H_2O_2 led to a significant increase in IL-8 amount during recovery reaching the highest value 456, 6±15,6 pg/ml at 24 h. The ultimate presence of IL-8 in the apical medium can resemble *in vivo* conditions, under which enterocytes produce cytokines directly into gut lumen.

In gastrointestinal tract the effective treatment alternative against excessive oxidative stress can be probiotics, which have been extensively used for maintenance of gut microbiota homeostasis, improvement of intestinal inflammation and other digestive disorders. The precise mode of action behind anti-inflammatory properties of probiotics remained to be

elucidated. Oxidative stress appears to be an important driving force for enhanced cytokine production in intestinal epithelial cell lines causing gut mucosal inflammation, thus the alteration in cytokine profile via probiotic therapy can lead to successful suppression of ROS-induced cellular damages [Matsumoto et al 2005, Sartor et al 2005, Watanabe et al 2009].

The presence of living probiotics is not prerequisite for decreasing secretion of proinflammatory cytokines. Previous study [Nemeth et al 2007, Malago et al 2010] revealed that SCS of *L. plantarum* 2142 significantly inhibited the synthesis of IL-8 induced by *Salmonella enteritidis* 857 in Caco-2 cells from the beginning to the end of recovery period (to 24 h) compared to that without SCS application. These findings were supported by previous results that SCS of *L. plantarum* 2142 could downregulate the effect of acute oxidative stress evoked by 1 h 1 mM H_2O_2 treatment on proinflammatory cytokine, IL-8 and TNF-α relative gene expression level in IPEC-J2 cells. A 1.8-fold decrease in IL-8 and 2.6 fold reduction in TNF-α level were detected when *L. plantarum* 2142 was used in 13.3 v/v% concentration [Paszti-Gere et al 2012].

Likewise, the level of protective Hsp70 increased significantly in the presence of *L. plantarum* 2142 supernatant by 2.7 fold compared to samples only treated with 1mM hydrogen peroxide. Interestingly, H_2O_2 treatment alone could also lead to elevation in Hsp70 relative gene expression in IPEC-J2 cells. The upregulation of Hsp70 expression can be one of the initial key defense mechanisms maintaining cell protective activity against inflammation-triggered protein misfolding defects. The increase in protective heat shock protein transcriptional level and remarkable drop in proinflammatory cytokine level support the efficacy of *L. plantarum* 2142 SCS against the oxidative stress-reduced inflammatory response. The mode of action behind the downregulation of elevated proinflammatory cytokine synthesis might include secreted bioactive substances or indirect action on protective Hsp70 production.

Enterococcus faecium is one of LA bacteria with inhibitory effects against several important enteropathogenics [Pollmann et al 2005]. An in vivo study on the effect of orally administered *Enterococcus faecium* EF1 on intestinal cytokines and chemokines production *in vivo* revealed that TNF-α production increased significantly in jejunal mucosa while it was suppressed in ileal mucosa in the newborn piglets treated with this probiotic strain [Huang et al 2012].

Anti-inflammatory effect of SCSs of *L. casei* Shirota was not significant, and those of other bacterial strains such as *Bifidobacterium animalis* subsp. *lactis* BB-12 and *E. faecium* CECT 4515 did not decrease peroxide-evoked TNF-α upregulation. Our experiments also

proved that the anti-inflammatory effect of SCS was not based on the ability of peroxide decomposition due to the assumed scavenging properties, since the peroxide amount did not change in solution with SCS and peroxide mixture compared to that containing peroxide alone.

It was found that levan is one of the potential immunstimulant components released into the supernatant by *Bacillus amyloliquefaciens* CECT 5940 equipped with levansucrase enzyme [Rairakhwada 2009]. The immunstimulant activity of levan appears to rely on induction of Toll-like receptor 4 (TLR-4) signalling partially through induction of IL-12 production [Xu et al 2006]. In our study, modulated response of proinflammatory cytokine network was achieved by SCS of *Bacillus amyloliquefaciens* which caused upregulation of the peroxide-induced IL-8 and TNF-α relative gene expression level in IPEC-J2 cells.

The fact that *L. plantarum* 2142 SCS alone can decrease the production of proinflammatory cytokines underlines the presence of active bacterial metabolites responsible for efficient quenching of oxidative stress-induced acute inflammatory responses. To find out which bioactive components are secreted by *L. plantarum* 2142 into SCS, two short-chain carboxylic acids were investigated whether they can downregulate peroxide-triggered elevated proinflammatory cytokine (IL-8 and TNF-α) levels as a consequence of their potential anti-inflammatory effect. LA production of *lactobacilli* is one of the main factor in regulating antibacterial activity. L-LA displays a greater antibacterial effect to D-LA or racemic LA. This study supported that neither acetic acid at 10 and 50 mM nor racemic, L- and D- LA in concentration range between 60-100 mM could prevent IPEC-J2 cells from presenting increased IL-8 and TNF-α level when exposed to oxidative stimuli. Anti-inflammatory effect of metabolites derived from two LABs (*Bifidobacterium breve* and *Streptococcus thermophilus*) was investigated in THP-1 cell line and peripherial blood mononuclear cells under LPS control. It was concluded that bioactive molecules from the chosen probiotics could eliminate LPS-induced TNF-α scretion in dose-dependent manner and they could increase the basal TNF- α production. These active compounds were non-protein type based on their resistance to pepsin-trypsin hydrolysis and molecular mass was lower than 3 kDa. LPS-induced TNF-α secretion was unchanged by millimolar LA alone [Menard 2004] in accordance with our findings.

We found that incubation of IPEC-J2 cells with sodium butyrate at 2 mM conferred cytoprotection against oxidative stress. It was proved that sodium butyrate at 2 mM concentration could increase TERs in IPEC-J2 cells in the presence of peroxide which elevation was significant even after 24 h recovery period. In vivo, butyrate feeding increased

the amount of *lactobacilli* and consequently the production of L-lactic acid significantly with concomitant reduction in pH. This is in good agreement with previous findings [Bokori and Gálfi 1989, 1990] which proved the significant negative correlation between the reduced number of coliform bacteria and elevation in lactobacilli count in swine ileum. Significantly higher amount of Ki-67 positive crypts in ileum was found in butyrate-fed pigs compared to controls suggesting elevated butyrate-induced cell proliferation extent. The number of microvilli on ileal mucosa was significantly higher when pigs were fed with diet containing 0.15% sodium butyrate. In butyrate-treated animals the number of microvilli was 27±3 per plica versus 19±2 per plica in controls (p<0.05). In accordance with our results it can be assumed that feed supplemented with sodium-n-butyrate exerts beneficial effect in the treatment of inflammatory bowel diseases in swine. The anti-inflammatory mode of action is based on the reinforcement of epithelial barrier and the maintenance of gut ecosystem via increasing the count of *lactobacilli* and the amount of protective metabolites in addition to butyrate protective effect against excessive oxidative stimuli. Therapeutical value of butyrate was further supported by significant enhancement of epithelial cell proliferation and elevated microvilli counts in swine ileum compared to untreated control values.

Among metabolites secreted by *L. plantarum* 2142, peptides can also exert protective action on the intestinal epithelium. In this study, to our knowledge for the first time molecular masses of two *L. plantarum* 2142-specific proteins (21 and 31 kDa) were detected by SDS-PAGE. By the aid of another separation technique, capillary zone electrophoresis the presence of another specific peptide was found in SCS of *L. plantarum* 2142.

In conclusion, in our work non-carcinogenic porcine intestinal epithelial cells, IPEC-J2 cells were cultured on collagen-coated polyester membrane inserts. This cell line with higher polarization rate and with apically located microvilli could mimic physiological conditions better thus it was found to be reliable screening tool for investigation of regulatory changes in cytokine network caused by excessive oxidative stress and for studying on modulatory effect of protective substances. *In vitro* models were optimized to be capable of screening the beneficial effect of probiotics either on prevention of intestinal epithelium from harmful oxidative stimuli or on restoration of physiological microbial communities in inflammatory processes. One of the prerequisites necessary for elucidation of the precise role of probiotic strains and their metabolites in acute inflammatory mechanisms was that even partial agonistic and antagonistic effect of probiotics could be traced on basal and elevated cytokine regulation. IPEC-J2 cells were proven to be suitable for investigation of both the immunstimulant and oxidative stress-modulating effect of selected probiotic strains.

The fact that spent culture supernatant of probiotics can decrease the production of proinflammatory cytokines suggests that active ingredient(s) can be found among *lactobacilli*-secreted metabolites responsible for the putative anti-inflammatory action against acute oxidative stress. Successful identification of these active bacterial components would enable the development of pharmaceutical products containing only the isolated beneficial substances with numerous advantages in contrast to drug formulations with living microorganisms. In living forms, probiotics should survive during storage, they should reach the GI tract without significant loss and they should adhere to mucous membrane with the ability for reproduction and pathogenic exclusion. These essential requirements have impact on mainly the manufacturing processes of probiotic containing animal feeds and pharmaceutical products. On the other hand, one of the most prominent milestones in pharmaceutical science would be the establishment of a targeted drug therapy with controlled release of active ingredient in solid formulations thereby ensuring higher bioavailability of probiotics with better pharmakokinetic profile. There are some innovative recent research works involving electrospinning-aided preparation of nanoformulations with lactobacilli [Wagner et al 2011].

It has not been fully understood yet whether single metabolite produced by probiotics or synergism between secreted molecules exerts anti-inflammatory action of bacterial supernatant. Among the investigated bacterial strains only SCS of *L. plantarum* 2142 had significant attenuating effect on peroxide-induced upregulation of IL-8 and TNF-α level. This beneficial effect can not be attributed to the presence of short chain carboxylic acids, lactic acid and acetic acid in the supernatant. The presence of *Lactobacillus plantarum* 2142-specific proteins was demonstrated by SDS-PAGE. In the future the aim will be isolation and characterization of the active components present in the SCS of *lactobacilli* and elucidation of the underlying mechanisms (signal transduction pathways and/or free radical scavenging effect) of protection against oxidative cell damage.

5. New scientific results

This is a pioneering study in which a porcine non-transformed intestinal epithelial cell line, IPEC-J2 was exposed to H_2O_2 alone or in combination with probiotics, and changes in TER and relative gene expression levels of proinflammatory cytokines (IL-8 and TNF-α) and Hsp 70 were measured with concomitant characterization of the anti-inflammatory *L. plantarum* 2142 supernatant. This is the first study in which TER values of IPEC-J2 cells were measured as related to ROS-induced oxidative stress on TJ protein profile (claudin-1, claudin-4 and claudin-7 and E-cadherin).

(1) We have shown that IPEC-J2 cell line cultured on collagen-coated polyester membrane inserts provides a reliable model for investigation of porcine-specific oxidative stress-induced inflammatory processes involving regulatory changes in the cytokine network.

(2) The maximal protection against 1mM H_2O_2–triggered inflammation was achieved using 13.3 v/v% *L. plantarum* 2142 as the gene expression level of TNF-α remained low as it was characteristic of 13.3v/v% MRS broth and SCS in the absence of peroxide. The protective properties of *L. plantarum* 2142 SCS can not be attributed to metabolite-triggered chemical decomposition of H_2O_2.

(3) *Bacillus amyloliquefaciens* CECT 5940 manifested in upregulated IL-8 and TNF-α relative gene expression level in IPEC-J2 cells.

(4) D-lactic acid, L-lactic acid and their racemic mixture in the concentration present in *L. plantarum* 2142 SCS did not reduce significantly peroxide-induced increase in IL-8 and TNF-α relative gene expression pointing out that the major downregulators of oxidative stress are not lactic acid in probiotic supernatant.

(5) Butyric acid at millimolar concentration contributed to enhanced enterocyte proliferation and stimulated formation of microvilli on plicae *in vivo*, and it was capable of suppression *E. coli* 30037 growth and at the same time it increased *lactobacilli* colony forming *in vitro*.

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7. List of publication

7.1. Original publications related to the PhD thesis

Paszti-Gere E., Csibrik-Nemeth E., Szeker K., Csizinszky R., Jakab Cs., Galfi P.: **Acute oxidative stress affects IL-8 and TNF-α expression in IPEC-J2 porcine epithelial cells,** Inflammation, 35 (3). 994-1004, 2012

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7.2. Additional publications

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7.4. Presentations at national conferences related to the PhD thesis

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"Struggling and suffering are the essence of a life worth living. If you're not pushing yourself beyond the comfort zone, if you're not demanding more from yourself - expanding and learning as you go - you're choosing a numb existence. You're denying yourself an extraordinary trip. If you can't run then walk. And if you can't walk, then crawl. Do what you have to do. Just keep moving forward and never ever give up." *"Karno"*