Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection

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ABSTRACT

This study aimed to examine the potential antiviral activity of lactic acid bacteria (LAB) using animal and human intestinal and macrophage cell line models of non tumor origin. To this end, LAB strains selected on the basis of previous *in vitro* trials were co-incubated with cell line monolayers, which were subsequently challenged with rotavirus (RV) and transmissible gastroenteritis virus (TGEV). In order to elucidate the possible mechanism responsible for the antiviral activity, the induction of reactive oxygen species (ROS) release as well as the attachment ability of LAB on the cell lines was investigated. Various strains were found to exhibit moderate to complete monolayer protection against viral RV or TGEV disruption. Highest protection effects were recorded with the known probiotics *Lactobacillus rhamnosus* GG and *Lactobacillus casei* Shirota against both RV and TGEV, while notable antiviral activity was also attributed to *Enterococcus faecium* PCK38, *Lactobacillus fermentum* ACA-DC179, *Lactobacillus pentosus* PCA227 and *Lactobacillus plantarum* PCA236 and PCS22, depending on the cell line and virus combination used. A variable increase (of up to 50%) on the release of NO^- and H_2O_2 (ROS) was obtained when LAB strains were co-incubated with the cell lines, but the results were found to be LAB strain and cell line specific, apart from a small number of strains which were able to induce strong ROS release in more than one cell line. In contrast, the ability of the examined LAB strains to attach to the cell line monolayers was LAB strain but not cell line specific. Highest attachment ability was observed with *L. plantarum* ACA-DC 146, *L. paracasei* subsp. *tolerans* ACA-DC 4037 and *E. faecium* PCD71. Clear indications on the nature of the antiviral effect were evident only in the case of the *L. casei* Shirota against TGEV and with *L. plantarum* PCA236 againt both RV and TGEV. In the rest of the cases, each interaction was LAB-cell line–virus specific, barring general conclusions. However, it is probable that more than one mechanism is involved in the antiviral effect described here. Further investigations are required to elucidate the underlying mode of action and to develop a cell line model as a system for selection of probiotic strains suited for farm animal applications.

1. Introduction

Animal diarrheal diseases represent a serious threat to farm animal welfare but also to the economic viability of animal husbandry. Since the ban of antibiotic growth promoters in the European Union (E.U.) in 2006 (Regulation 1831/2003/EC, 2005), probiotic animal feed supplementation has risen as a viable alternative to antibiotics, as reported for several monogastric or ruminant farm animals (Scharek et al., 2007; Pollmann et al., 2005; Stella et al., 2007; Maragkoudakis et al., submitted). Probiotics (Fuller, 1989; Guarner and Shaafsma, 1998) are comprised primarily of lactic acid bacteria which form part of the normal enteric flora of man and animals. Probiotic feed supplementation may benefit the animal host directly, by preventing the infection and combating the causative agent of the intestinal disorder, or indirectly, by balancing the disrupted equilibrium of the enteric flora and augmenting the host's immune responses.

Apart from bacteria, however, viruses are often the causative agent of farm animal diarrheal disease, including among them the transmissible gastroenteritis virus (TGEV) and rotavirus (RV). TGEV can cause al disease that has very high mortality rates (often 100%) in young piglets and is characterized by vomiting and severe diarrhea, while the symptoms manifest in a milder way in adult pigs with lower severity and mortality rates (Saif and Heckert, 1990). TGEV belongs to the family of coronaviruses and can been encountered globally where intensive pork industry is practiced, causing great economic losses (Sestak and Saif, 2002). RV on the other hand, a member of the *Reoviridae* family, is better known as being the leading cause of infant and young children diarrhea (Parashar et al., 2003) accounting for more than 500,000 deaths each year in children under five years (WHO epidemiological report, 2007). However, RV is also a common cause of diarrhea in a variety of farm animals such as lambs, calves and pigs (Holland, 1990; Saif and Heckert, 1990), in which the infection

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severity may range from asymptomatic to fatal, having severe economic implication in animal husbandry.

Although various literature and clinical studies have confirmed the beneficial and alleviating effects of probiotic bacteria, such as *Lactobacillus rhamnosus* GG, on the infection and symptoms of rotavirus diarrhea (Isolauri, 2003; Pant et al., 2007), no data is available on field studies of probiotic administration against viral enteric disorders of farm animals. Such studies can be limited by their complexity, as well as the ethical and economical implication of using valuable assets such as farm animals in viral diarrhea challenge studies. As an alternative, however, animal intestinal epithelial or macrophage cell line models can be developed and utilized, for studying the interaction between probiotics, viruses, and the host epithelium.

Very few attempts have been made only recently to study the impact and potential benefit of probiotic strains on animal cell lines. Nissen et al. (2009) studied the gut health promoting activity of putative probiotic strains, using pig intestinal and macrophage cell lines. Ivec et al. (2007) first reported a cell line model where probiotics were applied on pig epithelial cell lines to protect against vesicular stomatitis virus (VSV), followed by Botić et al. (2007) who reported similar results using probiotics to protect a porcine macrophage cell line against VSV disruption.

Addressing the scarce relevant literature, the aim of this study was to investigate *in vitro* the applicability of potential and established probiotic lactic acid bacteria as protective agents against the farm animal diarrheal viruses TGEV and RV. A pool of promising LAB strains, studied in detail for their probiotic properties by the E.U. funded project PathogenCombat (FP6-007081), has been included in this study. To assess the antiviral effects, various farm animal cell lines have been co-incubated with the LAB strains prior to their challenge with RV and TGEV viruses. Additional functional properties that relate to the possible underlying mode of action of the antiviral effect have been also studied, including release of reactive oxygen species (ROS) from the cell lines due to probiotic interaction, as well as the attachment ability of probiotic strains on the cell line monolayers.

2. Materials and methods

2.1. Bacteria and culture conditions

All LAB strains were cultured for routine use in de Man, Rogosa and Sharp broth (MRS, Oxoid, U.K.) for 18 h at 30 or 37 °C, depending on species. All strains (Table 1) belong to the collection of the European research project PathogenCombat (FP6-007081) and were drawn from the collections of the Agricultural University of Athens, Greece (PCA/ACA-DC), Danisco S/A, Denmark (PCD), Max Rubner Institute in Karlsruhe, Germany (PCK), and the University of Maribor, Slovenia (PCS). MRS agar (1.5% g/v, Oxoid, U.K.) was used for growth on solid media while enumeration of the strains was carried out by the standard serial dilution method, at 30 or 37 °C, according to species, for 48 h. All strains were stored at −80 °C in cryovials with MRS broth supplemented with 20% (v/v) of glycerol until use.

2.2. Selection of LAB strains used in this study

The strains applied on intestinal epithelial and macrophage cell lines in this study were selected on the basis of previous *in vitro* and *in vivo* work (Table 1) dealing with their probiotic and protective properties such antimicrobial activity against food spoilage, food and clinical pathogens, survival in simulated gastrointestinal tract and food processing conditions, as well as attachment on epithelial cell lines and induction of cytokine release from human macrophage cell lines.

2.3. Cell lines

The cell lines used comprised of intestinal epithelial and monocyte/macrophage derived cell lines of farm animal and human origin (Table 2).

Table 1
Lactic acid bacteria strains used in this study.

Designation	Species	Origin	Reference
ACA-DC 146	*L. plantarum*	Feta cheese	Maragkoudakis et al. (2006)
ACA-DC 179	*L. fermentum*	Kasseri cheese	Zoumpopoulou et al. (2008) Maragkoudakis et al. (2009)
ACA-DC 4037	*L. paracasei tolerans*	Kasseri cheese	Maragkoudakis et al. (2006)
BFE 900	*E. faecium*	Bean sprouts	Franz et al. (1999)
BFE 2207	*E. faecium*	Fermented food (Sudan)	Yousif et al. (2005)
BFE 5092	*L. plantarum*	Fermentd milk (Kenya)	Patrignani et al. (2006)
PCA 142	*L. fermentum*	Kasseri cheese	Nissen et al. (2009)
PCA 185	*L. gasseri*	Feta cheese	Nissen et al. (2009)
PCA 227	*L. pentosus*	Unknown	Unpublished PathogenCombat data
PCA 236	*L. plantarum*	Kaseri cheese	Unpublished PathogenCombat data
PCD 71	*Ent. faecium*	Sausage	Maragkoudakis et al. (2009)
PCS 20	*L. plantarum*	Slovenian cheese	Nissen et al. (2009) Schillinger and Villarreal (2010)
PCS 22	*L. plantarum*	Slovenian cheese	Nissen et al. (2009) Schillinger and Villarreal (2010)
PCS 25	*L. plantarum*	Slovenian cheese	Nissen et al. (2009)
PCS 26	*L. plantarum*	Slovenian cheese	Nissen et al. (2009)
LcS	*Lb. casei* Shirota	Human	Known probiotic
LGG	*L. rhamnosus* GG	Human	Known probiotic

All cell lines are available at the Dept. of Biochemistry, Faculty of Medicine, University of Maribor, Maribor, Slovenia. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Missouri, USA), supplemented with 10% Fetal Calf Serum (Biowhittaker, Maryland, USA), L-glutamine (2 mmol/l, Sigma-Aldrich), penicillin (100 units/ml, Sigma-Aldritch) and streptomycin (1 mg/ml, Sigma-Aldrich) at 37 °C in a humidified 5% CO_2 atmosphere in tissue culture flasks (Corning, USA) until confluent. The cell culture medium was regularly changed. To perform biological assays, the cells were seeded in 96-well plates at a concentration of 6×10^6 cells/ml and incubated for 24–48 h as described above until confluency. Just before use, the culture medium was removed and the monolayers were washed twice with DMEM without phenol red and supplements.

2.4. Virus

Transmissible Gastroenteritis Coronavirus (TGEV) and Rotavirus RF strain (RV) were used in this study. Both viruses were propagated in CLAB and PSI cells in the presence of trypsin (1 µg/ml of DMEM) as described previously (Botić et al., 2007). Supernatant containing the virus was collected from the flasks when cytopathic effect (CPE) was observed (24–48 h at 37 °C, 10% CO_2) by microscopy and collected by centrifugation at 3500 g for 10 min. Virus was stored at −70 °C until used. $TCID_{50}$ (Tissue culture infective dose) was determined exactly as previously described (Botić et al., 2007).

Table 2
Intestinal and monocyte/macrophage derived cell lines used in this study.

Cell line	Type	Organism	Source
H4	Epithelial	Human	University of Maribor
TLT	Monocyte/macrophage	Human	University of Maribor
PoM2	Monocyte/macrophage	Pig	University of Maribor
CLAB	Enterocyte like	Pig	University of Maribor
PSI	Epithelial	Pig	University of Maribor
GIE	Epithelial	Goat	University of Maribor

2.5. Cytopathic effect (CPE) reduction assay

The potential antiviral activity of probiotic bacteria was performed as described previously (Botić et al., 2007; Ivec et al., 2007). Initially, LAB strains were applied on the confluent monolayers as already described (Maragkoudakis et al., 2006). Briefly, overnight LAB cultures were harvested (3500 g, 10 min, 4 °C) and washed twice with PBS buffer, pH 7.2., before resuspension in non-supplemented DMEM to a concentration of 10^8 cfu/ml. The growth medium of 96-well plates was removed and the monolayers were washed twice with PBS. Subsequently, 100 µl of bacterial DMEM suspension was transferred onto the monolayers before incubation at 37 °C in a 5% CO_2 atmosphere for 90 min. Afterwards the non-bound bacteria were washed off with non-supplemented DMEM and TGEV or RV were applied (100 µl of 1.6 $TCID_{50}$/ml and 1.5 $TCID_{50}$/ml) on the monolayers. The plates were then incubated at the same conditions and monitored for signs of cytopathic effect (CPE), i.e. monolayer disruption, after 24 h and 48 h, as described previously (Botić et al., 2007; Ivec et al., 2007).

2.6. Determination of Reactive Oxygen species (ROS): NO and H_2O_2

For ROS determination, LAB strains were applied on healthy cell line monolayers as described above. The NO concentration was determined by measuring the accumulation of nitrate using a modified Griess reagent (Sigma), according to the Griess reaction as previously described (Ivec et al., 2007; Pipenbaher et al., 2009) by absorbance measurement at 540 nm The release of H_2O_2 was determined by transferring 50 µl of supernatant into a new 96-well plate and adding 50 µl of 0.01% peroxidase and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (diluted with distilled water 1:1), according to the instructions of the supplier (Pierce, Rockford, USA). After 15 min the reaction was stopped by addition of 50 µl H_3PO_4 and the absorbance was measured at 450 nm.

2.7. Determination of adhering LAB strains

LAB strains were grown, applied on confluent cell monolayers in 96-well plates and incubated as described above. Following incubation the medium supernatant with the non-attached bacteria was removed and the intestinal cell lines were washed twice with non supplemented DMEM. 100 µl of trypsin solution (Botić et al., 2007) were then added to detach adhered bacteria, which were subsequently enumerated by the standard serial dilution method on MRS agar plates at 30 or 37 °C, according to species, for 48 h, under microaerobic conditions (Anaerocult A, Merck, Germany).

2.8. Statistical analysis

Experimental data were analysed with the StatGraphics Centurion XV software (StatPoint Inc., USA) using the ANOVA multiple sample comparison. Statistical significant effects ($P < 0.05$) were further analysed and means were compared by the Tukey's HSD test.

3. Results

3.1. Antiviral effect of LAB

A selected number of LAB strains were first applied on the CLAB porcine epithelial cell line. The application of RV resulted in the complete disruption of the monolayer, with a survival percentage of only 10%. However, the co-incubation of the CLAB cell line with specific LAB strains resulted in increased survival percentages, from 40% up to 80% (Fig. 1). After this preliminary assay, selected LAB strains where then applied to four more intestinal epithelial and macrophage cell lines, which apart from RV, where also challenged with TGEV. The application of RV (Fig. 2a) and TGEV (Fig. 2b) on all cell lines tested led to a heavy disruption of the monolayers, with survival percentages of approx. 10% of that of the healthy confluent cell lines. As with the preliminary assay on CLAB cells, the pre-treatment with LAB strains prior to viral challenge led from small to marked increased survival percentages and a protective effect against monolayer disruption. In both cases of RV and TGEV, highest protection percentages were observed on all cases of viral challenge with *L. casei* Shirota and *L. rhamnosus* GG.

Specifically looking at both the preliminary and subsequent antiviral assays, in the case of the cell line challenge with RV, *L. rhamnosus* GG conferred a notable protective effect on monolayers against viral disruption, with survival ranging from $44.3 \pm 2.5\%$ (on H4) to $101.0 \pm 7.6\%$ (on PSI), i.e. complete protection. Other notable protection effects (>40% survival, i.e. four times higher than the survival obtained with the virus application) were observed with *E. faecium* PCK38, *L. fermentum* ACA-DC179 and *L. plantarum* PCS22 (on CLAB), *L. casei* Shirota (on CLAB and PSI) and *L. plantarum* PCA236 (on GIE).

Similarly, the application of *L. casei* Shirota conferred a protective effect against TGEV monolayer disruption, with cell line survival ranging from $41.2 \pm 2.6\%$ (on PSI) to $92.6 \pm 9.3\%$ (on GIE), i.e. almost complete protection, compared to healthy confluent monolayers. Notable protection effects (>40% survival) were also obtained with *L. rhamnosus* GG (on TLT, GIE and H4), *L. fermentum* ACA-DC 179 (on TLT), *E. faecium* PCD71 (on PSI), *L. plantarum* PCA236 (on GIE) and *L. pentosus* PCA227 (on H4). In all other cases, the application of LAB against viral disruption
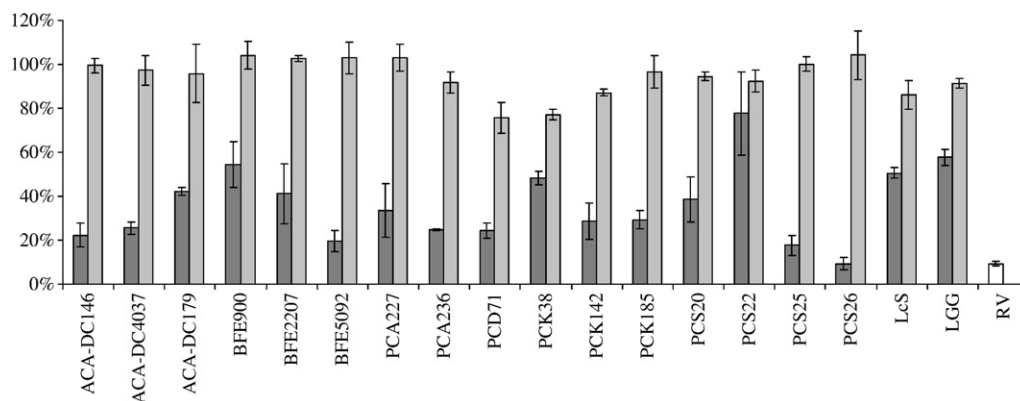

Fig. 1. Protective effect of selected lactic acid bacteria on intestinal CLAB cell line challenged with rotavirus (RV). Results are presented as % of monolayer integrity compared to a healthy growing cell line. □ — Monolayer challenged with RV, ▨ — monolayer pre-incubated with selected LAB strains and then challenged with RV, and ▨ — monolayer challenged with selected LAB strains. All values are expressed as mean ± standard deviation, two repetitions in triplicates.
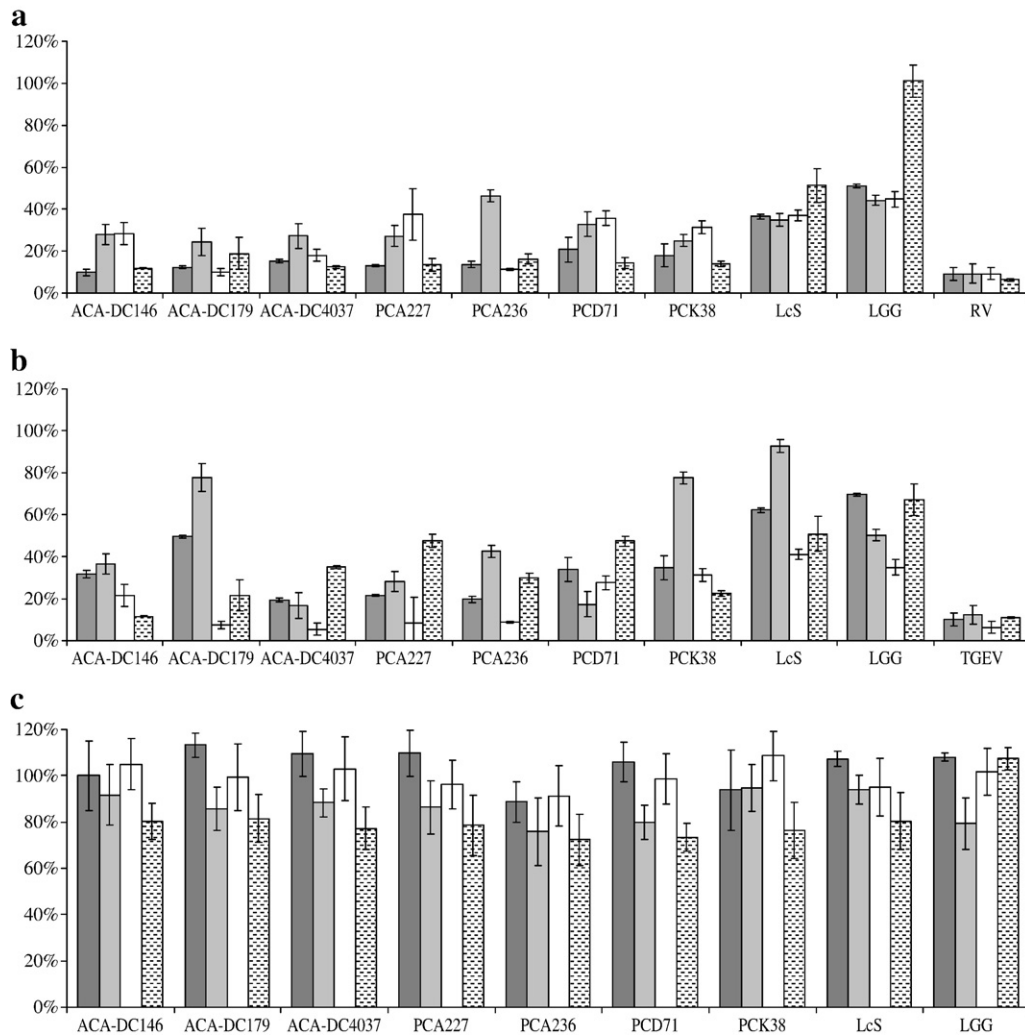
Fig. 2. Protective effect of selected lactic acid bacteria on intestinal (GIE — ▨, H4 — ☐ and PSI — ⊡) and macrophage (TLT — ▦) cell lines cell line challenged (a) with RV, (b) with TGEV. Results are presented as % of monolayer integrity compared to a healthy growing cell line. The survival of the cell lines at the presence of only the LAB strains are presented in (c). All values are expressed as mean ± standard deviation, two repetitions in triplicates.

of the cell line monolayers led to survival percentages less than <40%, i.e. from no protection at all to up to three times better survival, compared with that of the viral challenge.

In addition, the application of the LAB strains on the examined cell lines did not lead to any detrimental effects on the cell line integrity (Figs. 1 and 2c). Generally, the survival of the cell lines with the applied LAB strains ranged from 90–110%, compared to healthy monolayers, with the main exception evident in the case of the PSI cell line, that appears to be more sensitive to the addition of lactic acid bacteria, as a slightly lower survival percentage is observed (80%). Apart from the PSI cell line, similar slightly reduced survival can be observed in few additional cases, notably with GIE cell line (with *E. faecim* PCD71, *L. plantarum* PCA236 and *L.rhamnosus* GG) and the CLAB cell line (with *E. faecium* PCD71 and PCK38). However, statistical analysis revealed that significant differences (i.e. higher survival, $P<0.05$) exist only in the case of TLT cell line compared with GIE, CLAB and PSI as well as with H4 compared with GIE and PSI.

3.2. Nitric oxide (NO⁻) and hydrogen peroxide (H₂O₂) release

The release of NO⁻ and H₂O₂ from the various cell lines, due to co-incubation with LAB can be seen in Figs. 3 and 4. As before, a preliminary screening with selected strains was done on the CLAB cell line, before proceeding to screen more LAB strains on other cell lines.

The results obtained are LAB strain specific but also cell-line specific, as variable values are obtained when the same LAB strains are applied to difference cell-lines. None of the LAB strains adversely affected the release of NO⁻ in any of the cell lines tested. Instead, there was an increase in the NO⁻ production, ranging from 10–50%, which as previously, was LAB strain and cell line dependent. However, some trend lines could be observed, such as the increased release of NO⁻ by the CLAB, GIE, H4 and PSI cell lines stimulated by *L. plantarum* PCA236. Other strains, inducing an increased NO⁻ release (>20%), include *L. pentosus* PCA227 (on GIE and H4), *L. plantarum* ACA-DC 146 (on CLAB and PSI) as well as *L. casei* Shirota (on H4 and TLT). Results were even more variable in the case of H₂O₂, with secretion values that ranged from a slight decrease (20%) to a marked increase (50%) compared to the control. The only exception where a trend can be observed was in the case of the PSI cell line, in which the application of all LAB strains led to a decrease in the H₂O₂ release, ranging from 20 to 40%. As before, a trend could be observed in H₂O₂ release induction with strains *L. plantarum* PCA236 (on GIE and TLT), *L. plantarum* ACA-DC 146 (on H4 and TLT), as well as *L. casei* Shirota (H4 and TLT).

3.3. LAB adhesion to epithelial cell lines

Nine LAB strains were selected and applied on the intestinal epithelial cell lines H4, CLAB, PSI and GIE (Fig. 5). Since no strain
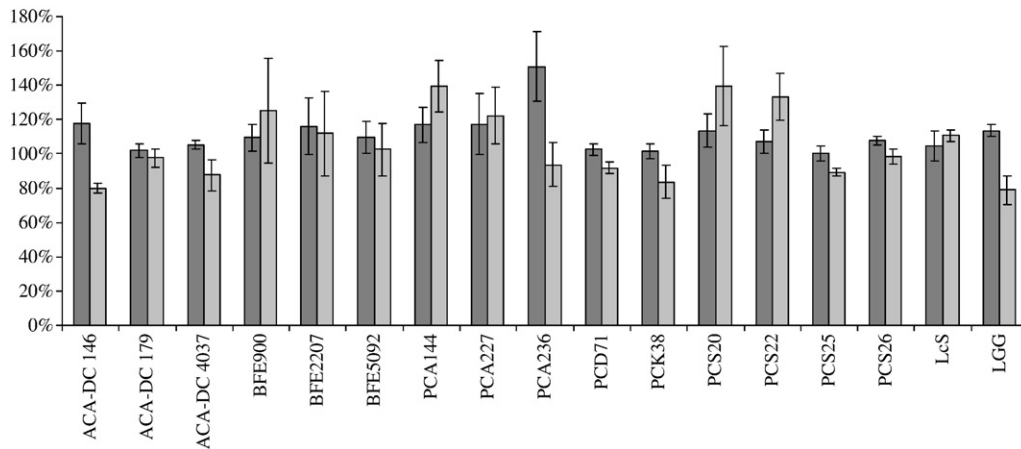
Fig. 3. Reactive oxygen species (ROS) release from the CLAB intestinal epithelial cell line, co-incubated with selected LAB strains. Results are predented as a % of release of NO^- (■) and H_2O_2 (▨) from the CLAB cell line without the presence of LAB, and expressed as mean ± standard deviation of two repetitions in triplicates.

variability was observed in the NO^- and H_2O_2 assays, the strains were selected on the basis of previously performed work. As can be observed, *L. plantarum* ACA-DC 146, *L. paracasei* subsp. *tolerans* ACA-DC 4037 and *E. faecium* PCD71 demonstrate increased attachment ability on four cell lines tested, ranging from 35 to 65%, with the only exception being *L. paracasei* subsp. *tolerans* ACA-DC 4037 on PSI cells (15% attachment). Although notable attachment ability was also demonstrated by *L. fermentum* ACA-DC 179, the rest of the strains tested exhibit lower attachment ability, ranging from 5–15%. In contrast to the NO^- and H_2O_2 experiment, the results obtained from

this assay are strain specific, as the attachment ability of the strains remained the same even on different intestinal epithelial cell lines, with no statistical differences observed on attachment of the strains between the different cell lines ($P > 0.05$).

4. Discussion

In humans, the alleviating effects of probiotic lactic acid bacteria on diarrheas associated with antibiotic therapy (Arvola et al., 1999; Vanderhoof et al., 1999) or acute rotavirus infection (Isolauri, 2003;
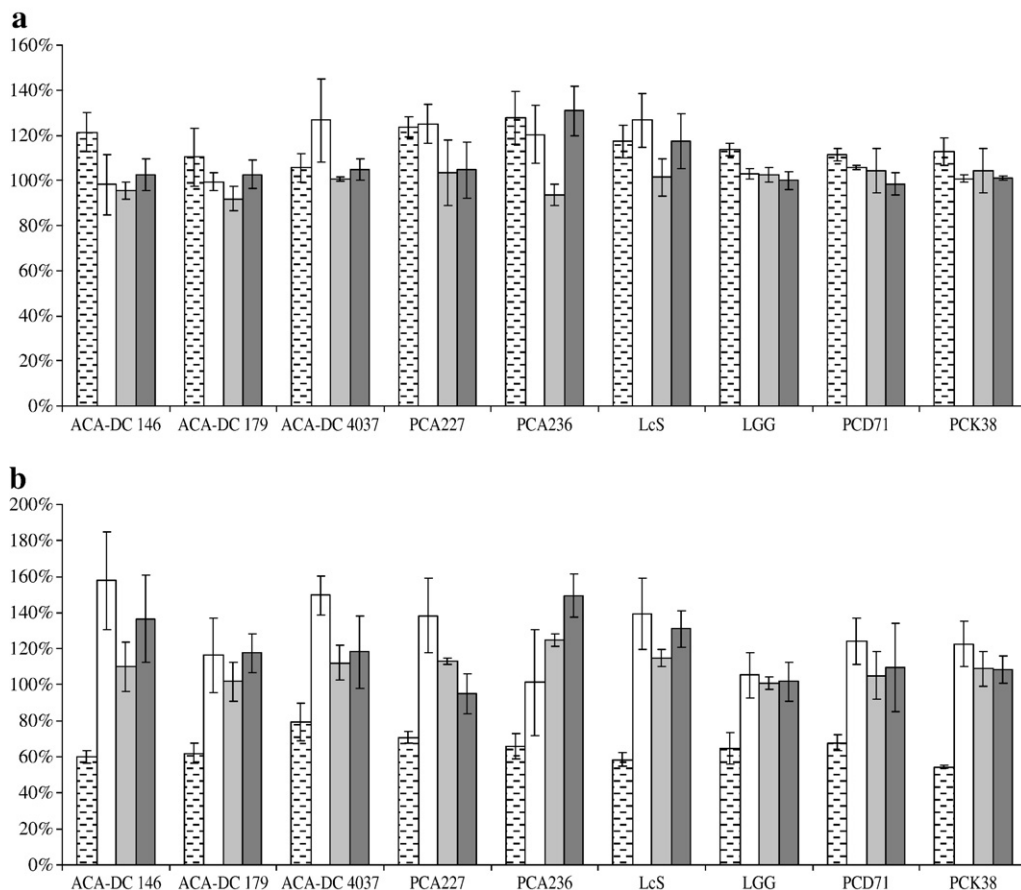

Fig. 4. Reactive oxygen species (ROS) release from (GIE — ▨, H4 — □ and PSI — ▨) macrophage (TLT — ■) cell lines incubated with selected LAB strains. Results are presented as a % of release of NO^- (a) and H_2O_2 (b) from the intestinal cell line without the presence of LAB, and expressed as mean ± standard deviation of two repetitions in triplicates.
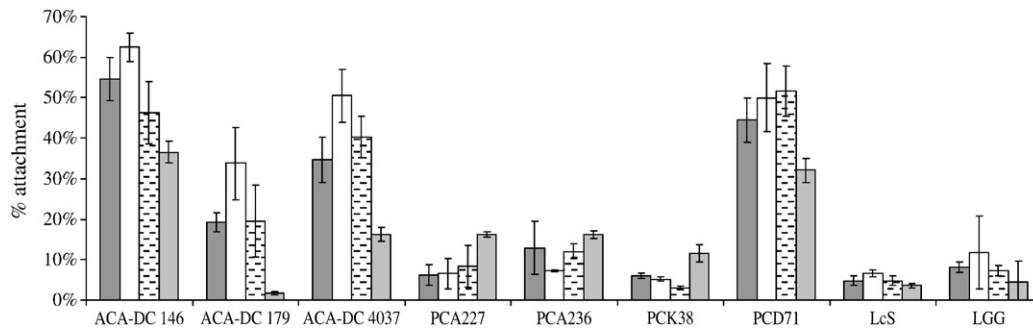
Fig. 5. Attachment of selected lactic acid bacteria strains on four intestinal epithelial cell lines, CLAB (■), H4 (□), GIE (▨) and PSI (▧). Results (mean ± standard deviation, two repetitions in duplicates) expressed as % of attached bacteria at the end of the assay to the initial number of bacteria applied on the cell lines.

Pant et al., 2007) diarrheas are nowadays well established and in fact constitute the major clinically proven health benefit of probiotics (Isolauri, 2003). In farm animals, some studies have demonstrated the efficacy of probiotic administration in reducing the incidence of diarrhea (Taras et al., 2006; Scharek et al., 2007), which, however, have not been associated with viruses.

Taking the above into account, as well as the important economic losses in animal husbandry due to TGEV and RV enteric disorders, this study aimed to examine directly the potential protective effect of selected LAB on animal and human cell lines challenged with viruses. The intestinal and macrophage cell lines used in this study were not of tumor origin, in order to simulate more closely the host–probiotic interactions, as previously reported (Pipenbaher et al., 2009). The cell lines were co-incubated with various LAB strains and challenged subsequently with TGEV and RV, in order to simulate a possible scenario of bacteria feed supplementation, where the probiotic organisms are already present in the intestinal lumen at the time of viral infection. For the preliminary antiviral assay, the intestinal porcine CLAB cell line was chosen, as it represented a good model for both viral infection and LAB adhesion, based on previous laboratory data (unpublished).

A LAB strain specific antiviral protective effect was observed in both preliminary CLAB assay and subsequent challenges of the other cell lines, with the known probiotics *L. casei* Shirota and *L. rhamnosus* GG, as well as *E. faecium* PCD71 and PCK38, *L. plantarum* PCS22 and PCA236 and *L. fermentum* ACA-DC179. Although both of the known probiotics used in this study have been used extensively in the literature, few cases have been reported on their antiviral effects in animals (Zhang et al., 2008) and, to the best of our knowledge, none have been reported on cell lines. In fact, very few data exists in general on the use of probiotics as viral infection deterrents on cell lines. Recent studies (Botić et al., 2007; Ivec et al., 2007) reported the protective effect that various lactic acid bacteria, including the probiotic *Lactobacillus paracasei* F19, conferred upon porcine intestinal epithelial and macrophage cell line infected with vesicular stomatitis virus (VSV). In the above studies, the antiviral effect mechanism was attributed to a variety of factors, including possible competition for attachment sites between probiotic bacteria and the virus and stimulation of innate cell responses or pro-inflammatory responses from the cell lines.

The elucidation of the underlying mechanism of action of the probiotic strains was attempted also in this study. To this end, we investigated the induction of reactive oxygen species NO⁻ and H_2O_2 release (ROS) by cell lines co-incubated with lactic acid bacteria. ROS has a variety of defensive roles in the host, such as killing of intracellular pathogens, tumor cells, but also virus-infected cells (Hibbs et al., 1998; Keyaerts et al., 2004). The antiviral effects of NO⁻ in particular have been previously reported (Bi and Reiss, 1995; Paludan et al., 1998; Ellermann-Eriksen, 2005). However, over expression of NO⁻, especially chronic, could have toxic side-effects also for the host cells (Brown, 2003). Another mechanism that could be involved in the anti viral effect is the competition for attachment sites on the epithelial

cell surfaces between probiotic bacteria and virus particles. This protective mode of action has already been cited in cell line interactions between probiotic bacteria and intestinal pathogens (Cocconier et al., 2000; Ouwenhand et al., 2001; Lievin-Le Moal et al., 2002; Maragkoudakis et al., 2006), and implied as a possible mechanism of protection against VSV cell line infection (Botić et al., 2007; Ivec et al., 2007).

In the present work the two strains that presented the most pronounced antiviral protection were *L. casei* Shirota and *L. rhamnosus* GG. In the case of *L. casei* Shirota, the high ROS release in the TLT cell line coincides with a high level of protection against TGEV in the same cell line. However, in all other cases with *L. casei* Shirota, no correlations can be found between ROS release and antiviral activity. In addition, the low attachment capacity of the strain, reported also elsewhere (Juntunen et al., 2001; Ouwenhand et al., 2001; Maragkoudakis et al., 2006) does not allow for direct linking of attachment ability and antiviral effect. Similar observations can be drawn with *L. rhamnosus* GG, which, in spite of a low ROS induction and attachment ability in all cell lines, was found to exert a major antiviral effect in both TGEV and RV, in all cell lines tested.

On the other hand, *L. plantarum* PCA236 seems to be a constitutive inducer of ROS release, as it was able to stimulate ROS release in, four out of five cell lines tested. In addition, on the goat epithelial cell line (GIE) strain PCA236 induced increase release of both NO⁻ and H_2O_2. This could explain the antiviral activity obtained on the GIE monolayers with the particular LAB strain, where a notable protective effect against both RV and TGEV was observed. The attachment ability of the same strain however appears to be rather low (~10%), and so it is unclear if adhesion could contribute to the antiviral effect. The high-attaching strain *L. plantarum* ACA-DC146, also able to induce increase ROS release in H4 and TLT, exhibited only low antiviral potential. Similarly, another strain with high-attachment ability, *E. faecium* PCD71, but no particular ROS release induction, exhibited only a mild antiviral effect on only once cell line (GIE).

In the present work, clear indications on the nature of the antiviral effect were evident only in the case of the *L. casei* Shirota against TGEV on the human macrophace cell line (TLT) and with *L. plantarum* PCA236 againt both RV and TGEV on the goat epithelial cell line (GIE). Each LAB-cell line–virus interaction is specific, barring the drawing of general and definite conclusions. It is however evident from the obtained results that probably more than one mechanism may be involved in the observed antiviral effect, as previously hypothesized in similar studies (Botić et al., 2007; Ivec et al., 2007). In addition, apart from ROS and attachment, the release of pro-inflammatory cytokines as interleukins (IL) 6 and 12 or the antiviral interferon-γ (IFN-γ), is an important mechanism by which probiotic bacteria may protect host cells (Miettinen et al., 1998; Hessle et al., 1999; Maassen et al., 2000; Ivec et al., 2007). The mechanism of action of the antiviral effects reported in this study needs to be further investigated and so a more detailed, holistic approach would need to be adopted, focusing less on the screening of LAB strains against various cell lines and viruses, and more on in depth studies of the phenomenon.

To conclude, this study reported for the first time, to the best of our knowledge, a protective effect of lactic acid bacteria against TGEV and RV on animal and human intestinal and macrophage cell lines of non tumor origin. Although preliminary, the results presented here are of particular importance and merit further investigation, as they can lead to development of specific *in vitro* models for selection of probiotic strains with antiviral effects. Carefully selected probiotics could then be applied as animal feed supplement in order to provide specific protection against subclinical or acute viral diarrheas or intestinal disorders in general in farm animals.

Acknowledgement

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