Arch Virol (2013) 158:799–807
DOI 10.1007/s00705-012-1543-0

ORIGINAL ARTICLE

Antiviral effects of a probiotic *Enterococcus faecium* strain against transmissible gastroenteritis coronavirus

Weidong Chai · Michael Burwinkel · Zhenya Wang · Christiane Palissa · Bettina Esch · Sven Twardziok · Juliane Rieger · Paul Wrede · Michael F. G. Schmidt

Received: 24 July 2012 / Accepted: 7 October 2012 / Published online: 28 November 2012

Abstract The enteropathogenic coronavirus transmissible gastroenteritis virus (TGEV) causes severe disease in young piglets. We have studied the protective effects of the probiotic *Enterococcus faecium* NCIMB 10415 (*E. faecium*), which is approved as a feed additive in the European Union, against TGEV infection. *E. faecium* was added to swine testicle (ST) cells before, concomitantly with, or after TGEV infection. Viability assays revealed that *E. faecium* led to a dose-dependent rescue of viability of TGEV-infected cells reaching nearly to complete protection. Virus yields of the *E. faecium*–treated cultures were reduced by up to three $\log_{10}$ units. Western blot analysis of purified TGEV revealed that the levels of all viral structural proteins were reduced after *E. faecium* treatment. Using transmission electron microscopy, we observed attachment of TGEV particles to the surface of *E. faecium* which might be a means to trap virus and to prevent infection. Increased production of nitric oxide in the cells treated with *E. faecium* and elevated expression of interleukin 6 and 8 pointed to stimulated cellular defense as a mechanism to fight TGEV infection.

W. Chai · M. Burwinkel (✉) · Z. Wang · C. Palissa · B. Esch · M. F. G. Schmidt
Institute of Immunology, Freie Universität Berlin, Philippstrasse 13, 10115 Berlin, Germany
e-mail: michael.burwinkel@fu-berlin.de

S. Twardziok · P. Wrede
Molecular Biology and Bioinformatics, Charité - Universitätsmedizin Berlin, Arnimallee 22, 14195 Berlin, Germany

J. Rieger
Institute of Veterinary Anatomy, Freie Universität Berlin, Koserstr. 20, 14195 Berlin, Germany

Introduction

Transmissible gastroenteritis virus (TGEV) infects enteric and respiratory tissues and causes severe gastroenteritis with a mortality rate close to 100 % in newborn piglets [3, 36]. The appearance of the closely related TGEV variant porcine respiratory coronavirus (PRCoV) has been found beneficial in preventing TGEV infections, possibly through induction of neutralizing antibodies that can provide cross-protection against TGEV infection [34, 39]. However, TGE prevalence is still being reported, and some TGEV strains have been isolated from domestic pigs in different parts of the world [28]. Commercially available vaccines, either inactivated or attenuated, have failed to provide full protection to piglets [38]. It is likely that the parentally applied inactivated viruses do not induce the local immune response in the small intestine that is required for protection. Therefore, the discovery and development of new, highly potent anti-TGEV agents and effective approaches for controlling the emergence of TGEV infection remains an important mission.

Probiotics are defined as live microbial food supplements with health-promoting attributes. Potent mechanisms of beneficial action include the production of antimicrobial agents, modulation of immune responses and promotion of host innate defense mechanisms [6, 7, 13, 20, 31]. *Enterococcus faecium* NCIMB 10415 (*E. faecium*) is authorized in the EU for use as a probiotic feed additive for sows and piglets and several other farm animal species. Beneficial effects of the probiotic *E. faecium* such as immune modulation and improvement of nutrient transport have previously been reported in several studies [12, 23, 32, 33, 37]. *In vitro* studies have also demonstrated that *E. faecium* could reduce the rate of invasion of pathogens—for instance, *Salmonella* in intestinal cell lines

[2, 9]. But detailed knowledge on the impact of *E. faecium* on viral infections *in vivo* and *in vitro* is lacking.

The purpose of the present study was to establish an *in vitro* model to investigate the antiviral potential of *E. faecium*. We used an established swine testicle (ST) cell line to assess the protective effects of *E. faecium* on TGEV infection in terms of viral replication and cell survival. To gain insight into its possible mechanisms of action, the effects of *E. faecium* on viral protein synthesis as well as the induction of inducible nitric oxide synthase (iNOS) and selected cytokines were investigated. Our results described here suggest that this probiotic *E. faecium* strain exhibits antiviral activity against TGEV and may possibly serve as a useful antiviral agent against coronavirus infections in vivo.

Materials and methods

Cells and virus

The epithelial swine testicle (ST) cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, PAN Biotech) supplemented with 10 % fetal bovine serum (Hyclone), and 1 % penicillin/streptomycin (Biochrom), growing at 37 °C in a 5 % CO_2 humidified incubator. The TGEV strain Purdue 46-MAD (kindly provided by Dr. C. Schwegmann-Wessels, Institut für Virologie, Tierärztliche Hochschule Hannover) was used in this study. Stock virus was propagated in ST cells to a titer of 1.00E+07 PFU/ml. All infections were done at a multiplicity of infection of 0.01.

Bacteria

Enterococcus faecium NCIMB 10415 isolated from a commercial product used in animal nutrition (Cylactin®, Cerbios-Pharma SA, Lugano) was used in this study and cultivated in Todd-Hewitt-Bouillon (THB, Roth). The number of viable bacteria in 1 ml of bacterial culture was determined by plating bacteria on agar. Bacterial cultures were then centrifuged at 2400 rpm for 10 min, and bacteria were washed twice to remove excess THB. Finally, the viable *E. faecium* particles were resuspended in DMEM to a stock concentration of 6.00E+08 CFU/ml.

Heat inactivation of bacteria was performed by heat treatment with *E. faecium* (1.00E+05, 1.00E+06, 1.00E+07 CFU/ml) in DMEM in a water bath at 100 °C for 10 min. Bacterial culture supernatants were obtained from growing bacterial cultures in THB. Bacteria were removed by centrifugation at 2400 rpm for 10 min, and supernatants were collected.

Assessment of cellular toxicity of *E. faecium*

Suspensions of 100 µl containing different amounts of *E. faecium* ranging from 1.00E+04 to 5.00E+08 were added to ST cell monolayers in a 96-well plate (Greiner Bio-One) for 1.5 h before washing away. At the end of the incubation period, a methylthiazolyl-diphenyl-tetrazolium bromide (MTT) viability assay was carried out as described previously [24]. The cell survival rate was determined as bacteria average OD value/control average OD value. The 50 % cytotoxic concentration (CC_{50}) was defined as the concentration that inhibited cell proliferation by 50 %, and a non-cytotoxic concentration of *E. faecium* was used for antiviral assays.

Impact of *E. faecium* on TGEV infection

Four different experimental protocols were applied to investigate the antiviral activity of *E. faecium*. Three setups focused on the effect of *E. faecium* on the cells by varying the treatment period in relation to infection with TGEV. A fourth setup assessed the direct effect of the probiotic on virus particles. In brief, monolayers of ST cells were treated with *E. faecium* for 1.5 h, which was washed away before infection with TGEV for 1 h (pretreatment assay), *E. faecium* and TGEV were added to the cell layer together during the 1-h infection period (competition assay), or *E. faecium* was added for 1.5 h right after the infection period (post-infection treatment assay). After probiotic treatment as well as after infection with TGEV, cells were washed twice and kept in medium containing 1 % penicillin/streptomycin to kill any viable bacteria that were left.

To assess direct effects of *E. faecium* on TGEV without cells being involved, the virus was mixed with different concentrations of *E. faecium* and incubated for 1.5 h at 37 °C. After centrifugation for 10 min at 5000 rpm to sediment bacterial cells, the virus containing supernatants were used to infect ST cells (cell-free preincubation assay).

The antiviral effects of heat-inactivated *E. faecium* as well as serially diluted *E. faecium* supernatants were also tested in the competition assay.

Virus-infected ST cells and cells without addition of *E. faecium* served as controls from which samples were collected at 48 and 72 h after infection (hpi) for the 50 % tissue culture infective dose ($TCID_{50}$) and the MTT viability assay, respectively. Relative survival of cells was calculated as follows [28]: Percent viable cells = [(OD value of *E. faecium* group − OD value of infection control)/(OD value of blank control − OD value of infection control)] × 100.

Transmission electron microscopy (TEM)

In order to examine possible direct binding of virus by *E. faecium*, the cell-free preincubation assay was performed by mixing *E. faecium* with TGEV at a bacteria-to-virus ratio of 500 for 1.5 h. After centrifugation for 10 min at 5000 rpm to sediment bacterial cells, the pellet was resuspended in 1 ml Karnovsky's Fixative. The samples were centrifuged for 10 min at 2500 rpm and a drop (15 µl) was taken from the bottom of the tube and negatively stained with 2 % phosphotungstic acid for 1 min. Finally, the samples were evaluated with a transmission electron microscope (Zeiss 10CR).

Virus yield reduction assay

ST cell monolayers were infected with TGEV with or without probiotic bacteria treatment according to the experimental design. At 48 hpi, aliquots of the supernatants were taken, and serial tenfold dilution steps were performed. Infectivity was determined by endpoint dilution titration on ST cells in a 96-well plate. The plate was incubated for 72 hpi, and infectivity was determined by recording the virus-induced cytopathic effect (CPE). Virus titer was calculated by the method of Reed and Muench, which is usually used for the calculation of LD_{50} [18] and documented as $TCID_{50}$ values.

Western blot analysis of virus particles

ST cells were infected in culture dishes with 145-cm^2 growth areas under competition assay conditions with *E. faecium*. At 48 hpi, virus from cell culture fluids and cells were collected by ultracentrifugation in a L7-65 Ultracentrifuge (Beckman Coulter) at 27,000 rpm for 2.5 h using a SW28 rotor. The pellet containing virus particles, with equal amounts of total protein, underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the separated proteins were electro-transferred to Hybond LFP (PVDF) membranes (GE Healthcare) using a feline anti-TGE polyclonal antiserum (NatuTec) at a dilution of 1:1000 and an anti-feline IgG polyclonal antiserum (Rockland) at a dilution of 1:10,000. Antigen-antibody complexes were detected using a western blotting substrate (Pierce® ECL Plus). Immunodetectable protein bands on the membrane were visualized using the Fusion SL4 imaging system (Vilber Lourmat), and protein amounts were estimated by densitometric analysis using the Fusion-Capt software (Vilber Lourmat). Three independent experiments and appropriate gel exposures yielded very similar results for each treatment modality.

Detection of nitric oxide (NO) release

At 48 hpi, in different assays, NO release was determined by measuring the amount of released NO_2^- using the Griess-Assay (Promega) according to the manufacturer's protocol. LPS (0.1 mg/ml)-stimulated cells were used as a positive control. Samples from untreated cells with or without prior infection served to define basal values.

Real-time PCR

Total RNA from ST cells was isolated using a Gene MATRIX RNA Purification Kit (EURx) as described by the manufacturer. Reverse transcription (RT) was performed using a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 25 µl in an iCycler iQ5 detection system (Bio-Rad Laboratories). Data analysis was based on the measurement of the cycle threshold (C_t). The differences in the Ct values of untreated samples versus treated samples were calculated by using the Delta-Delta-Ct method [22, 29]. Each sample was measured in triplicate from three independent experiments. The names of genes, the GenBank accession number, the primer sequences, the annealing temperatures, and the sizes of the amplification products are listed in Table 1.

Statistical analysis

All calculations were performed with IBM SPSS 20. Statistical analysis of virus titers and NO detection was performed by two and one factorial ANOVA, respectively, followed by Scheffé's post hoc test. Cytokine expression data analysis was performed by paired t-test. P-values less than 0.05 were considered statistically significant. All data are given as the mean ± SD.

Results

Assessment of cytotoxicity of *E. faecium* for ST cells

Before the probiotic *E. faecium* can be used for interference studies, the concentration range in which its addition to cells is non-toxic was defined. The results from cell viability assays (Fig. 1) show that *E. faecium* was non-toxic at concentrations up to 1.00E+07 CFU/ml. The viability rate of ST cells was 100 %, and no morphological differences were observed between bacteria-treated and mock-treated cells at this concentration. Therefore, the highest concentration of *E. faecium* chosen for the interference study with TGEV was 1.00E+07 CFU/ml. The CC_{50} of *E. faecium* in ST cells was calculated to be 5.92E+07 CFU/ml.

Table 1 Detailed primers and conditions used for real-time PCR assays

Gene	Accession number	Primer pairs (5′-3′)	Annealing temp. (°C)	Amplicon size (bp)
β-actin-for	XM_003124280.2	GGACTTCGAGCAGGAGATGG	55	233
β-actin-rev		GCACCGTGTTGGCGTAGAGG		
IL-1β-for	NM_214055	GGCCGCCAAGATATAACTGA	57	70
IL-1β-rev		GGACCTCTGGGTATGGCTTTC		
IL-2-for	EU139160	GCTGGATTTACAGTTGCT	55	213
IL-2-rev		CTTGTTTCAGATCCCTTT		
IL-6-for	AB194100	AACGCCTGGAAGAAGA	53	229
IL-6-rev		AACCCAGATTGGAAGC		
IL-8-for	X61151	GTTCTGGCAAGAGTAAG	53	275
IL-8-rev		CACGGAGAATGGGTTT		
IL-10-for	EF433759	GCATCCACTTCCCAACCA	55	178
IL-10-rev		TCGGCATTACGTCTTCCAG		
IFN-α-for	NM_214393	GCTCCTGGCACAAATG	60	197
IFN-α-rev		GCTGCTGATCCAGTCC		
TNF-α-for	NM_214022	ACGCTCTTCTGCCTACTGC	58	388
TNF-α-rev		TGGGCGACGGGCTTATC		
TLR-3-for	DQ266435	AACCAGCAACACGACT	57	110
TLR-3-rev		TTGGAAAGCCCATAAA		
TGEV-for	DQ811789	GTATTGGGATTATGCT	55	258
TGEV-rev		GGTGGTGGTAGTAGGT		

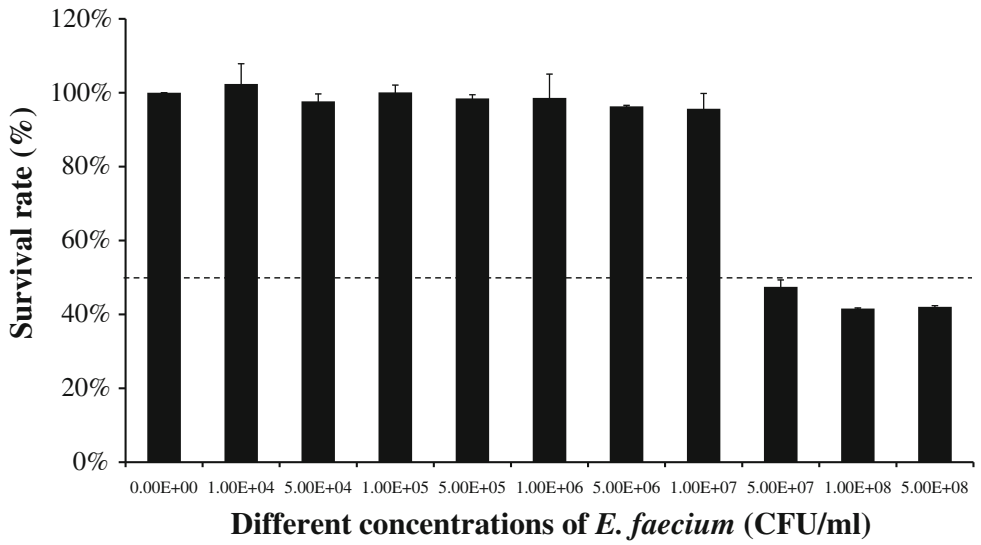

Fig. 1 Effect of *E. faecium* on the viability of ST cells. *E. faecium* was added to confluent cells in a 96-well plate, which were then incubated at 37 °C for 1.5 h. Cell viability was tested by MTT assay after 72 h. The cell survival rate was determined by comparing the optical density values from *E. faecium*–treated cells to those from non-treated control cells set to 100 %. The cell survival rates at different concentrations of probiotic bacteria are given, and 50 % above the cell survival rate (above broken line) is regarded as a non-toxic concentration of *E. faecium*. Results represent means ± standard deviations from three independent experiments

Assessment of protective effect of *E. faecium* during infection of ST cells with TGEV

Infection of cell cultures with TGEV is known to cause a severe CPE. If treatment of the host cells with *E. faecium* has a protective effect, it should cause a decrease in the virus-induced CPE, which should result in the rescue of the viability of the infected cells. The viability of the TGEV-infected cells was analyzed by both MTT assay and flow cytometry. The results from the MTT analysis (Fig. 2a) show that *E. faecium* provided protection from TGEV infection in a dose-dependent manner. Up to 100 % protection was achieved at the highest concentration of *E. faecium* (1.00E+07 CFU/ml) when the probiotic was added to the cells together with the virus during the infection period (competition assay).

To find out whether *E. faecium* inactivates TGEV particles by direct physical interaction with virus, a cell-free preincubation assay was performed. The results show that the infectivity of TGEV was also reduced in a concentration-dependent manner. These results from the MTT assay (Fig. 2a) were confirmed by flow cytometry using propidium iodide staining in an independent experiment (data not shown). Furthermore, as illustrated by electron microscopy (Fig. 3), virus particles seemed to be bound by *E. faecium* and attached to the *E. faecium* surface.

Because the competition assay exhibited the most pronounced antiviral activity in terms of cell survival (Fig. 2a), the antiviral effect of heat-killed *E. faecium* and *E. faecium* supernatant in the competition assay was also tested. The results (Figs. 2b, c) show that heat-killed *E. faecium* and *E. faecium* supernatant still had antiviral activity, but it was much less pronounced, suggesting that live *E. faecium* is necessary to exhibit the observed virus-reducing effects.

Effect of *E. faecium* on virus yields in TGEV-infected ST cells

The anti-TGEV activities of *E. faecium* were confirmed by measuring released infectious virus in the culture medium using a $TCID_{50}$ assay. As expected, the results (Fig. 4) are consistent with those from the cell viability assays, since TGEV yields were found to be reduced by treatment with *E. faecium*. Again, the inhibition of virus production was most effective in the competition assay, when cells had been exposed to the highest concentration of *E. faecium* (1.00E+07 CFU/ml), amounting to a three-$\log_{10}$ reduction. Reduced virus titers were also found in the cell-free preincubation assay, which indicates that the probiotic *E. faecium* also has antiviral capacity at the level of direct physical interaction with virus particles.

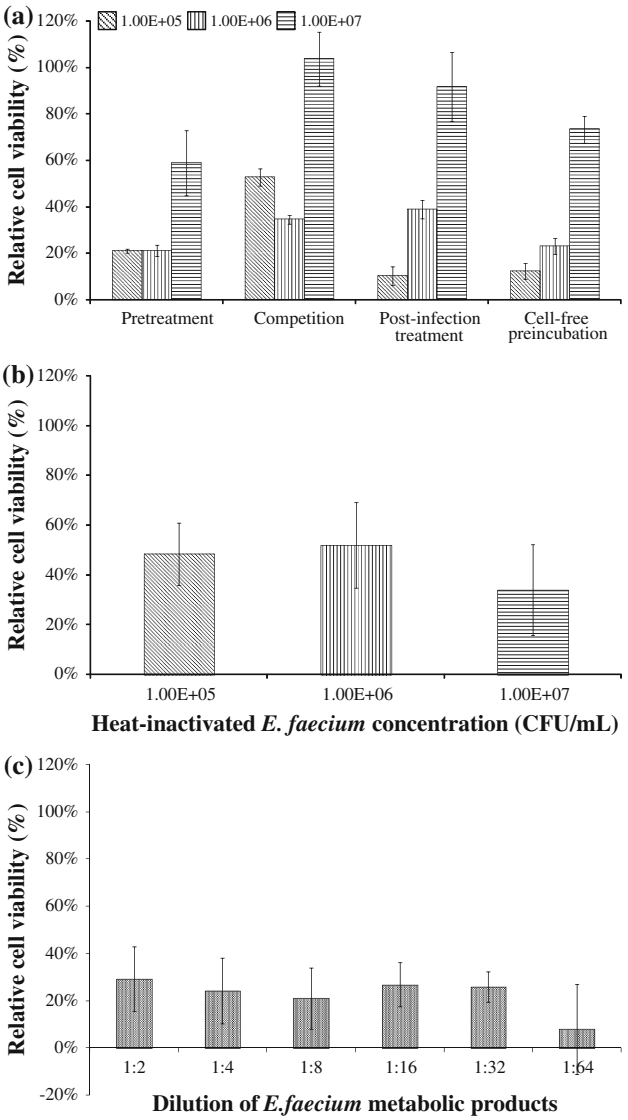

Fig. 2 Rescue of TGEV-infected ST cells by treatment with *E. faecium*. Different concentrations of *E. faecium* were added to ST cells in different setups as described (**a**). Heat-inactivated *E. faecium* (**b**) and diluted supernatants of cultured *E. faecium* (**c**) were also included. After 72 h, an MTT assay was carried out. Results are plotted as percent viability, with uninfected cells without *E. faecium* taken as 100 %. Results are given as mean ± standard deviation from at least three independent experiments

Analysis of protein composition of TGEV from ST cells treated with *E. faecium*

TGEV produced on a large scale under *E. faecium* interference conditions (competition assay) was enriched by ultracentrifugation and subjected to SDS-PAGE followed by western blot analysis. Protein assays revealed strongly reduced amounts of total viral protein when virus from probiotic treated cells was analyzed (Fig. 5). When virus was collected from cells treated with 1.00E+07 CFU/ml of

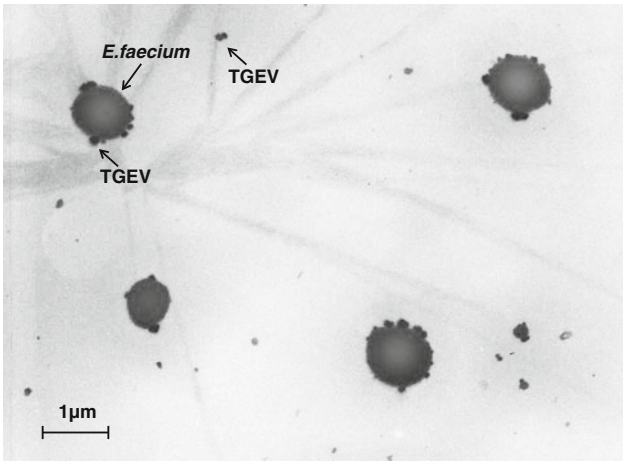

Fig. 3 Attachment of TGEV particles to *E. faecium.* The pellet of virus and bacteria mixture from cell-free preincubation assay was negatively stained and examined by TEM

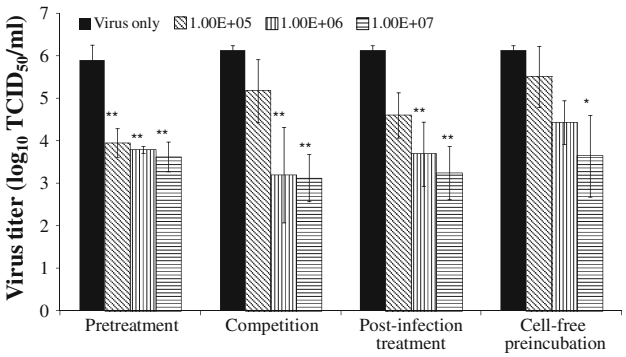

Fig. 4 Less virus produced in *E. faecium*–treated ST cells infected with TGEV. Cells were exposed to different concentrations of *E. faecium* as described. Cell culture supernatants were collected, and the yield of virus was determined by $TCID_{50}$. The means ± standard deviations from three independent experiments are shown. Significance levels for the difference between *E. faecium* treatment and virus control from untreated cells are given above the bar: **p < 0.001, *p < 0.05

the probiotic during the infection period, TGEV protein levels were reduced by more than 80 %. More importantly, after western blotting with antibodies raised against total TGEV protein, densitometric inspection failed to show any major aberrations of the relative polypeptide compositions of the virus particles, indicating that the levels of all viral proteins were evenly reduced.

E. faecium treatment increases the production of NO in ST cells

In a first approach to elucidating the mechanism of the effect of probiotic treatment on TGEV production, the synthesis of antiviral NO was measured. As shown in Table 2, all of the three different concentrations of

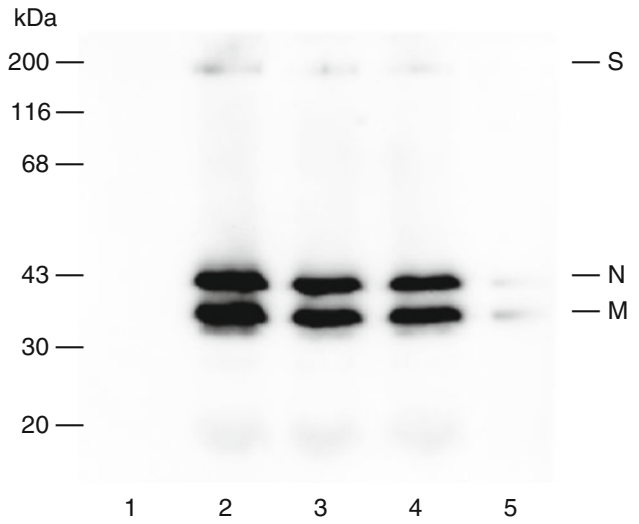

Fig. 5 Disrupted TGEV protein expression in *E. faecium*–treated ST cells infected with TGEV. Lane 1, uninfected, untreated cell control; lane 2, TGEV control; lane 3, virus from cells treated with 1.00E+05 CFU/ml *E. faecium*; lane 4, virus from cells treated with 1.00E+06 CFU/ml *E. faecium*; lane 5, virus from cells treated with 1.00E+07 CFU/ml *E. faecium.* Molecular weight marker proteins were run in parallel. Positions of viral spike protein (S), viral nucleocapsid protein (N), and viral membrane protein (M) are indicated on the right. Three independent experiments yielded almost identical results

Table 2 Effect of *E. faecium* on NO (μM NO_2^- ± SD) release in control ST cells and TGEV-infected ST cells

Additions to culture medium	Non-infected cells	TGEV-infected cells
No additions	0.20 ± 0.11	2.38 ± 0.39
E. faecium (1.00E+07)	5.92 ± 0.28**	6.67 ± 0.94**
E. faecium (1.00E+06)	4.92 ± 0.37**	5.30 ± 0.60**
E. faecium (1.00E+05)	4.06 ± 0.34**	4.49 ± 0.22*
LPS	4.80 ± 0.11**	ND

TGEV and *E. faecium* were present on ST cells for 1 h during the infection period (Competition assay). LPS at 100 μg/ml final concentration was present for 1 h in non-infected cells. Griess assay for NO detection was done 48 h after treatment. OD values at 540 nm were measured and nitrite concentration was calculated according to nitrite standard reference curve. Results are given as nitrite concentration and represent the means ± standard deviations from three independent experiments. Significance levels for the difference between the value for *E. faecium* and LPS treatment and those for the "no additions" in the same column are marked

*p < 0.05

**p < 0.01

E. faecium apparently induced NO release whether the cells had been infected with TGEV or not. The highest accumulation of NO in the cell culture medium was obtained in the 1.00E+07 CFU/ml *E. faecium*/virus group, exceeding

the levels of the positive control LPS, which is a strong inducer of NO release.

Cytokine expression in *E. faecium*–treated ST cells during the period of infection

Cytokines are important components of cellular defense mechanisms against microbial infection. Treatment of ST cells with the probiotic could modulate the cellular expression patterns of cytokines and thereby reduce the efficiency of TGEV multiplication. As a first step to test this hypothesis, we studied the production of selected cytokines under the influence of *E. faecium* in TGEV- and mock-infected ST cells at 2 h, 4 h, 6 h, 12 h and 24 hpi (competition assay). A clear increased expression of cytokines was observed, reaching the highest levels of expression at 4 hpi. The results show that administration of 1.00E+07 CFU/ml *E. faecium* together with the virus significantly increases mRNA expression levels of the pro-inflammatory cytokines interleukin 6 (IL-6) and IL-8 (an approximately 3- and 13-fold increase, respectively) when compared with TGEV-infected ST cells that had not been exposed to the probiotic (Fig. 6). Tumor necrosis factor-alpha (TNF-α), interferon α (IFN-α) and Toll-like receptor-3 (TLR-3) mRNA expression showed a less pronounced increase when compared with TGEV-infected cells that had not been exposed to the probiotic. Administration of 1.00E+07 CFU/ml *E. faecium* alone increased similar mRNA expression levels of those cytokines. IL-1β, IL-2 and IL-10 levels were apparently below the detection limit of the PCR assay applied in this study.

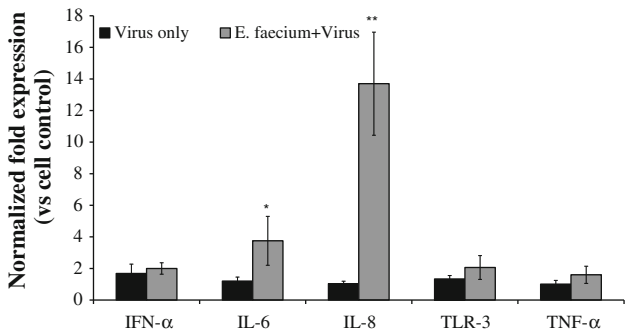

Fig. 6 Stimulation of cytokine expression by *E. faecium* in TGEV-infected ST cells. The expression of selected cytokines was measured by quantitative RT-PCR. The expression of IL-6 and IL-8 was significantly increased compared to the cells infected with TGEV only (black bars). Significance levels for the difference between *E. faecium* treatment and virus control are given above the bar: *$p < 0.05$, **$p < 0.01$. The data presented correspond to the mean ± SD of at least three independent experiments

Discussion

To assess the potential prophylactic or therapeutic effect of the probiotic bacteria *E. faecium* on TGEV infection, increasing concentrations of probiotic *E. faecium* bacteria were added to ST cells before, concomitantly with, or after TGEV infection for a short period of time, and cell viability as well as virus titers in the culture medium were quantitatively assessed later after long-term incubation. A low MOI of 0.01 was chosen in order to allow multiple infection cycles, as this more closely reflects natural infection.

By pre-treatment of ST cells with *E. faecium* (pretreatment), the viability of TGEV-infected cells was protected, and virus yields were reduced (Figs. 2 and 4). It appears that *E. faecium* can interfere with virus attachment and/or entry into cells. Several studies have demonstrated that probiotics can block viral attachment by competitive inhibition if they are able to bind viral receptors at the surface of cells. Freitas and coworkers [10, 11] reported that the *Lactobacillus casei* strain DN114001 and a strain of *Bacteroides thetaiotaomicron* produce a soluble compound that partially protects epithelial cells from rotavirus infection *in vitro* by modulating the apical glycosylation pattern of the cells.

The post-infection treatment assay suggested that the antiviral activity of *E. faecium* also contributed to the stimulation of pro-inflammatory factors (i.e., increased mRNA expression levels of IL-6 and IL-8). Pagnini and coworkers have shown that the multiple probiotic formulation VSL#3 could stimulate the epithelial production of TNF-α and activate NF-κB *in vitro* [26]. Probiotic bacteria may also indirectly interfere with virus by altering the state of cells, stimulating innate and/or adaptive immunity [5, 6]. In this study, the expression of antiviral cytokines IL-6 and IL-8 may alter the state of cells, eventually leading to an antiviral response.

In our cell-free pre-incubation assay, improved survival and a significant drop in virus titer were also observed (Figs. 2 and 4). In theory, the virus could also fail to infect the host cells if it is trapped by adsorption to the bacterial surface, and from the TEM result (Fig. 3), we did observe that virus particles were trapped by *E. faecium*. There may be some molecular mimicry between a bacterial surface molecule (such as glycoprotein with sialic acid) and a eukaryotic cellular receptor used by a virus for attachment [6].

In this study, the competition assay in which virus and probiotic bacteria are present in the culture medium side by side, exhibited the most pronounced antiviral activity in terms of cell survival (Figs. 2 and 4). This most pronounced antiviral activity most likely resulted from the sum of overlapping mechanisms at different time points before and after virus infection, as shown before, including

interference with the adsorption of virus to the host cells, trapping of virus particles or inhibiting the effective adsorption of the virus to the target cells, and the stimulation of pro-inflammatory factors.

The finding that the levels of all of the viral structural proteins were equivalently reduced after *E. faecium* treatment (Fig. 5) indicates that indeed fewer TGEV particles were released from these infected cells. Likewise, reduced synthesis of TGEV proteins may decrease the amount of virus-induced damage and subsequently also ameliorate the cytopathic effect in virus-infected ST cells, which logically must lead to a rescue of cell viability.

As the competition assay was the most effective antiviral approach, we looked for possible direct mechanisms for the effect of probiotic treatment by looking for NO release from infected cells. We found that *E. faecium* could significantly induce NO release (Table 2). The antiviral effects of NO have been well studied for several viral infections [1, 8, 15–17]. Although NO production is believed to be released mainly in macrophages, we did detect an increase of NO release in ST cells upon treatment with *E. faecium* and TGEV, and this release of NO was dose dependent. This indicates that an induction of iNOS could indeed play a role in the mechanisms for our observation that *E. faecium* inhibits TGEV infection in ST cells. For the competition assay, we also compared expression of the antiviral cytokines IL-6 and IL-8. TGEV infection of ST cells without *E. faecium* did not significantly increased expression when compared to mock control. However, *E. faecium* treatment significantly increased the production of pro-inflammatory factors IL-6 and IL-8 in TGEV-infected ST cells. This result is consistent with those of other authors [14, 19, 25, 30, 40], who showed IL-6 or IL-8 production following the interaction of probiotics with the intestinal epithelium. Because IL-6 and IL-8 responses in intestinal epithelial cells play important roles in the pathogenesis and immune defense against enteric pathogens [21], the increased level of those cytokines could also possibly indicate an enhanced innate response.

According to scientific opinion, probiotic concentrations between 10^6 and 10^8 CFU/g of intestinal contents are required to elicit potential benefits to the host [4, 27]. In feeding trials with piglets, concentrations of 10^6 - 10^7 CFU *E. faecium*/g digesta could be detected in the intestine [35]. Thus, although not directly comparable, the effective concentrations of *E. faecium* used in the present study are in a similar range, which adds to the relevance of these data.

In conclusion, the results of the present study show that *E. faecium* inhibits TGEV replication in ST cells and that possibly overlapping mechanisms lead to the observed reduction of virus growth: direct interference with virus attachment, adsorptive trapping or inactivation of virus particles through surface components of the probiotic bacteria, and the stimulation of pro-inflammatory cytokines IL-6 and IL-8 as well as NO production. The data suggest that *E. faecium* may serve as a useful antiviral agent against infection with TGEV and possibly other viruses. Challenge experiments with different porcine viruses in piglets are under way to substantiate this hypothesis.

Acknowledgements The study was funded by the Deutsche Forschungsgemeinschaft (DFG) through grant SFB 852/1 to sub-project A5 (MFGS).

References

1. Akerstrom S, Mousavi-Jazi M, Klingstrom J, Leijon M, Lundkvist A, Mirazimi A (2005) Nitric oxide inhibits the replication cycle of severe acute respiratory syndrome coronavirus. J Virol 79:1966–1969
2. Alexopoulos C, Georgoulakis IE, Tzivara A, Kritas SK, Siochu A, Kyriakis SC (2004) Field evaluation of the efficacy of a probiotic containing Bacillus licheniformis and Bacillus subtilis spores, on the health status and performance of sows and their litters. J Anim Physiol Anim Nutr (Berl) 88:381–392
3. Cavanagh D (1997) Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 142:629–633
4. Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. J Appl Microbiol 84:759–768
5. Clavel T, Haller D (2007) Molecular interactions between bacteria, the epithelium, and the mucosal immune system in the intestinal tract: implications for chronic inflammation. Curr Issues Intest Microbiol 8:25–43
6. Colbere-Garapin F, Martin-Latil S, Blondel B, Mousson L, Pelletier I, Autret A, Francois A, Niborski V, Grompone G, Catonnet G, van de Moer A (2007) Prevention and treatment of enteric viral infections: possible benefits of probiotic bacteria. Microbes Infect 9:1623–1631
7. Delcenserie V, Martel D, Lamoureux M, Amiot J, Boutin Y, Roy D (2008) Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol 10:37–54
8. Ellermann-Eriksen S (2005) Macrophages and cytokines in the early defence against herpes simplex virus. Virol J 2:59
9. Fairbrother JM, Nadeau E, Gyles CL (2005) Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim Health Res Rev 6:17–39
10. Freitas M, Cayuela C, Antoine JM, Piller F, Sapin C, Trugnan G (2001) A heat labile soluble factor from bacteroides thetaiotaomicron VPI-5482 specifically increases the galactosylation pattern of HT29-MTX cells. Cell Microbiol 3:289–300
11. Freitas M, Tavan E, Cayuela C, Diop L, Sapin C, Trugnan G (2003) Host-pathogens cross-talk. Indigenous bacteria and probiotics also play the game. Biol Cell 95:503–506
12. Ganesh BP, Richter JF, Blaut M, Loh G (2012) Enterococcus faecium NCIMB 10415 does not protect interleukin-10 knock-out mice from chronic gut inflammation. Benef Microbes 3:43–50
13. Gill HS (2003) Probiotics to enhance anti-infective defences in the gastrointestinal tract. Best Pract Res Clin Gastroenterol 17:755–773
14. Haller D, Bode C, Hammes WP, Pfeifer AM, Schiffrin EJ, Blum S (2000) Non-pathogenic bacteria elicit a differential cytokine

response by intestinal epithelial cell/leucocyte co-cultures. Gut 47:79–87
15. Ivec M, Botic T, Koren S, Jakobsen M, Weingartl H, Cencic A (2007) Interactions of macrophages with probiotic bacteria lead to increased antiviral response against vesicular stomatitis virus. Antiviral Res 75:266–274
16. Jung K, Gurnani A, Renukaradhya GJ, Saif LJ (2010) Nitric oxide is elicited and inhibits viral replication in pigs infected with porcine respiratory coronavirus but not porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 136:335–339
17. Kidd P (2003) Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 8: 223–246
18. Krah DL (1991) Receptors for binding measles virus on host cells and erythrocytes. Microb Pathog 11:221–228
19. Lammers KM, Helwig U, Swennen E, Rizzello F, Venturi A, Caramelli E, Kamm MA, Brigidi P, Gionchetti P, Campieri M (2002) Effect of probiotic strains on interleukin 8 production by HT29/19A cells. Am J Gastroenterol 97:1182–1186
20. Lebeer S, Vanderleyden J, De Keersmaecker SC (2010) Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 8:171–184
21. Liu F, Li G, Wen K, Bui T, Cao D, Zhang Y, Yuan L (2010) Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics. Viral Immunol 23: 135–149
22. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25:402–408
23. Lodemann U, Hubener K, Jansen N, Martens H (2006) Effects of *Enterococcus faecium* NCIMB 10415 as probiotic supplement on intestinal transport and barrier function of piglets. Arch Anim Nutr 60:35–48
24. Meshitsuka S, Ishizawa M, Nose T (1987) Uptake and toxic effects of heavy metal ions: interactions among cadmium, copper and zinc in cultured cells. Experientia 43:151–156
25. Otte JM, Podolsky DK (2004) Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. Am J Physiol Gastrointest Liver Physiol 286:G613–G626
26. Pagnini C, Saeed R, Bamias G, Arseneau KO, Pizarro TT, Cominelli F (2010) Probiotics promote gut health through stimulation of epithelial innate immunity. Proc Natl Acad Sci USA 107:454–459
27. Reid G, Sanders ME, Gaskins HR, Gibson GR, Mercenier A, Rastall R, Roberfroid M, Rowland I, Cherbut C, Klaenhammer TR (2003) New scientific paradigms for probiotics and prebiotics. J Clin Gastroenterol 37:105–118
28. Ren X, Meng F, Yin J, Li G, Li X, Wang C, Herrler G (2011) Action mechanisms of lithium chloride on cell infection by transmissible gastroenteritis coronavirus. PLoS One 6:e18669
29. Rothfuss O, Gasser T, Patenge N (2010) Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach. Nucleic Acids Res 38:e24
30. Ruiz PA, Hoffmann M, Szcesny S, Blaut M, Haller D (2005) Innate mechanisms for Bifidobacterium lactis to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats. Immunology 115:441–450
31. Salminen S, Nybom S, Meriluoto J, Collado MC, Vesterlund S, El-Nezami H (2010) Interaction of probiotics and pathogens—benefits to human health? Curr Opin Biotechnol 21:157–167
32. Scharek L, Guth J, Reiter K, Weyrauch KD, Taras D, Schwerk P, Schierack P, Schmidt MF, Wieler LH, Tedin K (2005) Influence of a probiotic *Enterococcus faecium* strain on development of the immune system of sows and piglets. Vet Immunol Immunopathol 105:151–161
33. Scharek L, Guth J, Filter M, Schmidt MF (2007) Impact of the probiotic bacteria *Enterococcus faecium* NCIMB 10415 (SF68) and *Bacillus cereus* var. toyoi NCIMB 40112 on the development of serum IgG and faecal IgA of sows and their piglets. Arch Anim Nutr 61:223–234
34. Schwegmann-Wessels C, Herrler G (2006) Transmissible gastroenteritis virus infection: a vanishing specter. Dtsch Tierarztl Wochenschr 113:157–159
35. Simon O (2010) An interdisciplinary study on the mode of action of probiotics in pigs. J Anim Feed Sci 19:230–243
36. Sola I, Castilla J, Pintado B, Sanchez-Morgado JM, Whitelaw CB, Clark AJ, Enjuanes L (1998) Transgenic mice secreting coronavirus neutralizing antibodies into the milk. J Virol 72: 3762–3772
37. Szabo I, Wieler LH, Tedin K, Scharek-Tedin L, Taras D, Hensel A, Appel B, Nockler K (2009) Influence of a probiotic strain of *Enterococcus faecium* on *Salmonella enterica* serovar Typhimurium DT104 infection in a porcine animal infection model. Appl Environ Microbiol 75:2621–2628
38. Tuboly T, Yu W, Bailey A, Degrandis S, Du S, Erickson L, Nagy E (2000) Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. Vaccine 18:2023–2028
39. Wesley RD, Lager KM (2003) Increased litter survival rates, reduced clinical illness and better lactogenic immunity against TGEV in gilts that were primed as neonates with porcine respiratory coronavirus (PRCV). Vet Microbiol 95:175–186
40. Zhang L, Li N, Caicedo R, Neu J (2005) Alive and dead *Lactobacillus rhamnosus* GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells. J Nutr 135: 1752–1756