2.0. MATERIALS AND METHODS

2.1. Materials

2.1.1. Experimental animals

Hybrid sows of German Landrace x Duroc and their piglets from the Institute of Animal Nutrition, Freie Universität Berlin.

2.1.2. Microorganisms

2.1.2.1. Probiotic: *Enterococcus faecium* SF68 (NCIMB 10415)

The supplemented probiotic strain, *E. faecium* NCIMB 10415, also referred to as *E. faecium* SF68 (Männer and Spieler, 1997), was used in a microencapsulated form of a commercial batch of the EC authorized probiotic feed additive Cylactin (Cerbios-Pharma, batch No. AG0551, Barbengo, Switzerland) and was mixed with the diet and pelleted at 50°C. The probiotic strain does not have resistance to vancomycin; and like the other strains of the same species, it is also sensitive to benzylpenicilline, aminopenicilline, imipenem, tetracycline and chloramphenicol.

2.1.2.2. *Salmonella typhimurium* DT 104

An isolate of multi-resistant *S. typhimurium* DT104 strain (BB 440) obtained from a swine with sepsis was used for animal challenge. The strain was additionally provided with a nalidixic acid resistance and the green fluorescent protein (GFP)-expressing plasmid pFVP25.1 carrying kanamycin resistant gene and with no impact on virulence. *S. typhimurium* DT104 was cultured as described in Szabó et al. (2009). The strain has been shown to be resistant against ampicillin, chloramphenicol, tetracycline, streptomycine, sulphonamide and spectinomycin. Each piglet was infected with 2ml of bacterial culture (ca. 3 x 10^{9}/ml) mixed with 8ml buffered peptone water (BPW) (1.12535. Merck, Darmstadt, Germany) diluted 1:10.

2.1.2.3. Transmissible gastroenteritis virus (TGEV)

TGEV was grown in the known model epithelial cell lines for studies on TGEV, epithelial McClurkin swine testis (ST) cell line, which were obtained from Professor G. Herrler, Institute of Virology, Hannover, Germany.

2.1.3. Reagents

Substance	Supplier	Based in
Agarose	QIAGEN	Hilden, Germany
Azaperon	Janssen-Cilag	Neuss, Deutschland
BD-FACS-Flow	BD Biosciences	Heidelberg, Germany
Bovine serum Albumin, Nr. A7030	Sigma-Aldrich	Steinheim, Germany
Brain Heart Infusion (BHI) Broth	Oxoid LTD	Basingstoke, Hampshire, England
Buffered peptone water (BPW) 1.12535	Merck	Darmstadt, Germany
Cacodylate buffer	Merck Eurolab, GmbH	Darmstadt, Germany
di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	Carl-Roth	Karlsruhe, Germany
DL-Dithiothreitol (DTT)	Sigma- Aldrich	Taufkirchen, Germany
DEPC	Sigma- Aldrich	Deisenhofen, Germany
DMEM	Pan Biotech	Aidenbach, Germany
DMSO	Sigma-Aldrich	Deisenhofen, Germany
dNTP Set, 100mM solution	Bioline	Luckenwalde Germany
EDTA	Sigma-Aldrich	Taufkirchen, Germany
Epon	Serva Electrophoresis GmbH	Heidelberg, Germany
Ethanol ≥ 99.8%	Carl-Roth	Karlsruhe, Germany
Ethidium bromide solution (10mg/ml)	QIAGEN	Hilden, Germany
FACS flow sheath fluid BD	Biosciences	Heidelberg, Germany
Fetal calf serum (FCS)	BioWhittaker	Berlin, Germany
Ficoll-Plaque plus 17-1440-02	Amersham Biosciences	Freiburg, Germany
Fornaldehyde solution (4% in PBS)	Carl-Roth	Karlsruhe, Germany
Gentamycin (10 mg/ml)	Sigma	Deisenhofen
Glycerin	Merck Eurolab, GmbH	Darmstadt, Germany

Hank´s Buffered Salt Solution (HBSS), Ca^{2+} and Mg^{2+} free, 10543-F	Cambrex Biosciences	Belgium
HyperLadder IV DANN Marker	Bioline	Luckenwalde, Germany
Ketamin	Janssen-Cilag	Neuss, Deutschland
ß-Mercaptoethanol	Merck Eurolab, GmbH	Darmstadt, Germany
Methylthiazol-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich	Steinheim, Germany
MACS MicroBeads	Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
PCR reaction components	Bioline	Luckenwalde Germany
Penicillin/ Streptomycin	Pan Biotech	Aidenbach, Germany
Pentobarbital	Essex Pharma	München, Germany
Percoll 17-0891-01, density 1,13 g/ml	Amersham Biosciences	Freiburg, Germany
Potassium Dihydrogen Phosphate (KH_2PO_4),	Carl-Roth	Karlsruhe, Germany
PrepProtect™ stabilization buffer	Miltenyi Biotec GmbH	Germany
Propidium iodide (PI) Nr. P4170, 95% (HPLC), Nr. 904153	Sigma-Aldrich	Steinheim, Germany
RPMI-1640-Medium, with L-Glutamin, phenol red, 12-702-F	Biochrom AG, Seromed	Berlin, Germany
Sodium carbonate (Na_2CO_3)	Carl-Roth	Karlsruhe, Germany
Sodium chloride	Carl-Roth	Karlsruhe, Germany
Sodium dodecyl sulphate (SDS)	Sigma Aldrich	Steinheim, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Merck	Darmstadt, Germany

TAE (Tris-acetate-EDTA) buffer (10x)	Carl-Roth	Karlsruhe, Germany
Trypan blue solution (0.4 %) Nr. T8157	Sigma Aldrich	Steinheim, Germany
Trypsin-EDTA	Biochrom AG, Seromed	Berlin, Germany

2.1.4. Kits

Method	**Product´s name**	**Supplier**	**Based in**
RNA Extraction	Invisorb Spin Cell RNA Mini Kit	Invitek	Berlin, Germany
RT PCR	cDNA Synthesis Kit	Fermentas	St. Leon-Rot, Germany

2.1.5. Enzymes

Method	**Product**	**Supplier**	**Based in**
RNA Extraction	RNase-free Dnase Nr. 74124	Qiagen GmbH	Hilden, Germany
DNA degradation	DNase I	Invitrogen GmbH	Karlsruhe, Germany
Real-time PCR	2x SensiMix (dT)	Quantace	Berlin, Germany
Reverse transcriptase	MMLV Reverse Transkriptase	Fermentas	St. Leon-Rot, Germany

2.1.6. Antibodies

Antibody	**Supplier**	**Based in**
Mouse anti-porcine CD4a, clone 74-12-4, conjugated to FITC	Biozol Diagnostika	Eching, Germany
Mouse anti-porcine CD8a, clone PT8, conjugated to R-phycoerythrin (R-PE)	Biozol Diagnostika	Eching, Germany
Mouse Anti-Human Cytokeratin (pan), Isotyp IgG1 Klon AE1/AE3	Serotec	Düsseldorf, Germany
Mouse Anti-Human CD45 clone MAC323	Biozol Diagnostika	Eching, Germany
Mouse anti-porcine CD14 Clone MIL-2	Serotec	Düsseldorf, Germany
Mouse Anti-porcine Vimentin Isotyp IgG1	Serotec	Düsseldorf, Germany

Polyclonal rabbit anti-human von Willebrand factor (VWF) glycoprotein	Dako	Denmark
Mouse anti-porcine CD31 (PECAM-1)	Serotec	Düsseldorf, Germany

2.1.7. Consumables

Material	Supplier	Based in
Cell culture plates	Greiner Bio-One	Frickenhausen, Germany
Cell scraper	Greiner Bio-One	Frickenhausen, Germany
Cover slips	Menzel	Braunschweig, Germany
Coulter clenz, an all-purpose cleaning reagent, Nr. 8448222	Beckman Coulter	Krefeld, Germany
Falcon, Chamber slides 8 chamber polystyrene vessel	Becton Dickinson Labware	Claix, France
Hand counter Nr. 880117509	NeoLab	Berlin, Germany
Hemocytometer	Carl-Roth	Karlsruhe, Germany
Improved counting chamber, Nr. 318-100-20232	Mercateo	Germany
Latex gloves	Carl-Roth	Karlsruhe, Germany
MiniMACS™ Separator	Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
PCR tubes	Eppendorf	Hamburg, Germany
Pipettes	Carl-Roth	Karlsruhe, Germany
Pipette Tips	Biozym Scientific GmbH	Hess. Oldendorf, Germany
Plastic Pasteur pipettes Nr. 4894.1	Carl-Roth	Karlsruhe, Germany
Real time PCR 96-well plates	Bio–Rad Laboratories	München, Germany
Spinner flasks Wheaton	Science Products	NJ, USA
Sterile filter (0.22 µm)	Millipore SA	Molsheim, France
Syringe	B.Braun	Melsungen, Germany

2.1.8. Instruments

Material	Supplier	Based in
Autoclave, Varioklav	H + P Labortechnik GmbH	Oberschleißheim, Germany
Centrifuge, Heraeus Labofuge 400R, 75008162;		Germany
Cold centrifuge-5417 R	Eppendorf,	Germany
Electron microscope (10CR)	Zeiss	Jena, Germany
ELISA plate reader	Tecan, GmbH	Germany
Flow Cytometer Beckman Coulter EPICS XL-MCL with 488nm-Argon-Laser		Oakville, Canada
H-600 Wilo-Prax Microscope		Bonn, Germany
Incubator shaker, Certomat IS	Vitaris AG, Baar	Switzerland
iQ5 real time PCR detection system	Bio-Rad Laboratories	München, Germany
Micropipetts	Eppendorf	Germany
Microplate Reader/washer	Bio-Tek Instruments GmbH	Bad Friedrichshall, Germany
Multi-Detektions Mikroplatten Reader Synergy™HT	Bio-Tek Instruments GmbH	Bad Friedrichshall, Germany
NanoDrop Spectrophotometer ND – 1000	PEQLAB Biotechnologie	Erlangen, Germany
PCR-Mastercycler Gradient	Eppendorf	Hamburg, Germany
Sensitive balance	Sartorius AG	Göttingen, Germany
Vortex-Typ Vortex Genie 2	scientific industries Inc.	Bohemia, NY, USA
Water bath, WNB	Memmert GmbH	Schwabach, Germany

2.1.9. Solutions

Collection medium

Hanks balanced salt solution (HBSS) without Ca2+ and Mg 2+ with gentamicin (50 mg/100ml of HBSS) and penicillin-streptomycin (2000 IU/2mg in 100ml of HBSS).

DTT/HBSS

HBSS without Ca2+ and Mg 2+

2mM DTT

0.01mM Hepes

EDTA/HBSS

HBSS without Ca2+ and Mg 2+

1mM EDTA

1mM Hepes

Erythrocytes lysis buffer

KH_2PO_4	0.01g/l
EDTA	0.0037g/l
NH_4Cl_2	0.83g/l
Aqua dest.	Add to 100ml

Phosphate buffer solution (PBS)

NaCl	8g/l
KCl	0.2g/l
KH2PO4	0.2g/l
Na2HPO4 x H2O	1.35g/l
Aqua dest	add to 1000ml

Cacodylate buffer, 0.2 M

Dissolve 8.56g $Na(CH_3)_2AsO_23H_2O$ (Merck Eurolab, GmbH, Darmstadt) in aqua dest to a final volume of 200ml and adjust the pH to 7.2-7.4 with 0.5M HCl

Cacodylate buffer, 0,1 M, pH 7.2

50ml cacodylate buffer (0.2M) plus 50ml aqua dest

Epon (All substances from Serva Electrophoresis GmbH, Heidelberg)

Epon-Glycidether 23.10g

dodecenyl succinic anhydride (DDSA) 14.25g

Methyl Nadic Anhydride (MNA) 12.55g

2,4,6-tris(dimethylaminomethyl)phenol (DMP-30) 0.75ml

6x loading buffer

4g sucrose, 25mg bromophenol blue, 2.4ml of 0.5M EDTA (for agarose gel) 10ml volume

Alginate dissolving solution

NaCl, sodium citrate pH 6.8

TE buffer

10mM Tris HCl pH 8.0, 1mM EDTA

50x Tris – Acetate – EDTA – buffer (1L)

242g Tris pH 8.5

57.1ml Vinegar

100ml 0-5M EDTA

2.2. *In-vivo* evaluation of the effect of *E. faecium* SF68 against *S. typhimurium* DT 104

2.2.1. Experimental design

This study was part of the interdisciplinary research project of the Deutsche Forschungsgemeinschaft (DFG), integrative analysis of the mechanism of action of probiotic *E. faecium* NCIMB 10415 (Cylactin) in swine. The animals were taken care of at the Institute of Animal Nutrition of the Faculty of Veterinary Medicine. Infection with *Salmonella typhimurium* DT 104 and slaughtering of the animals took place at the Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BFR).

The study used Landrace × Duroc sows and their litters. Ten days before parturition, sows were randomly assigned to two groups, consisting of an untreated control group receiving normal feed and a probiotic group, receiving feed supplemented with a microencapsulated preparation of *E. faecium* SF68 (NCIMB 10415) (Cylactin). Litter size within each treatment group was adjusted to meet an exclusion criterion of at least 9 but not more than 15 living piglets. Piglets of both the probiotic group (N = 43) and the control group (N = 46) were weaned at 28 days of age and reared together with their littermates. Sows and piglets of both treatment groups were strictly separated in housing facilities with identical constructional and environmental conditions. All animals were proved to be negative for *Salmonella* and the absence of the probiotic strain in animals of the control group was confirmed in random fecal samples of sows and piglets as well as intestinal contents of piglets. The application of prophylactic and therapeutic antibiotics to sows and their litter was prohibited. The lighting program was 16h light and 8h of darkness. The room temperature and relative humidity were adjusted to 21°C and 65%, respectively.

2.2.2. Animal feed

The feed for the sows was based on barley and wheat while piglets received diet based on wheat and soybean powder. Sows were fed twice a day and the amount of feed given to pregnant sows was dependent on their body weight while that of the lactating sows was depending on the litter size of the sows. Sows of the probiotic group (N = 10) were fed a diet supplemented with *E. faecium* SF68 (Cylactin) beginning from 25 days after mating, with continued feed supplementation during lactation. Gestation sows that were lactating did not receive probiotic in their diet. After weaning (day 28), the starter diet for piglets was with or without probiotic supplementation according to the treatment group. Piglets of the probiotic group received supplemented starter feed from days 29 to 56 of age. The mean concentrations of the supplemented *E. faecium* in the feed for gestation sows, lactating sows, nursed piglets and weaned piglets of the probiotic group were 2.2 x 10^6 (0.92 SD), 1.4 x 10^6 (0.57 SD), 7.5 x 10^6 (3.1 SD) and 4.4 x 10^6 (2.7 SD) viable cells per gram of feed respectively.

2.2.3. Infection with *S. typhimurium* DT 104

On the day of weaning, piglets were transported to the Federal Institute for Risk Assessment, where infection with *S. typhimurium* DT 104 took place. Piglets of both groups were challenged with 10^9 CFU *Salmonella* per pig on day 29 post-partum (p.p) by intra-gastric application using a stomach tube. Each piglet was sedated by intramuscular application with 1.0 mg/kg azaperon prior to infection. To obtain tissue samples from the gut immune system, two piglets from each litter were randomly selected and sacrificed 3h, 24h and 71h post infection (p.i), whereas the remaining animals were monitored for a period of four weeks and sacrificed at day 28 p.i (Figure 4). The number of piglets slaughtered at each time point and their respective ages are listed on Table 1.

Experimental design

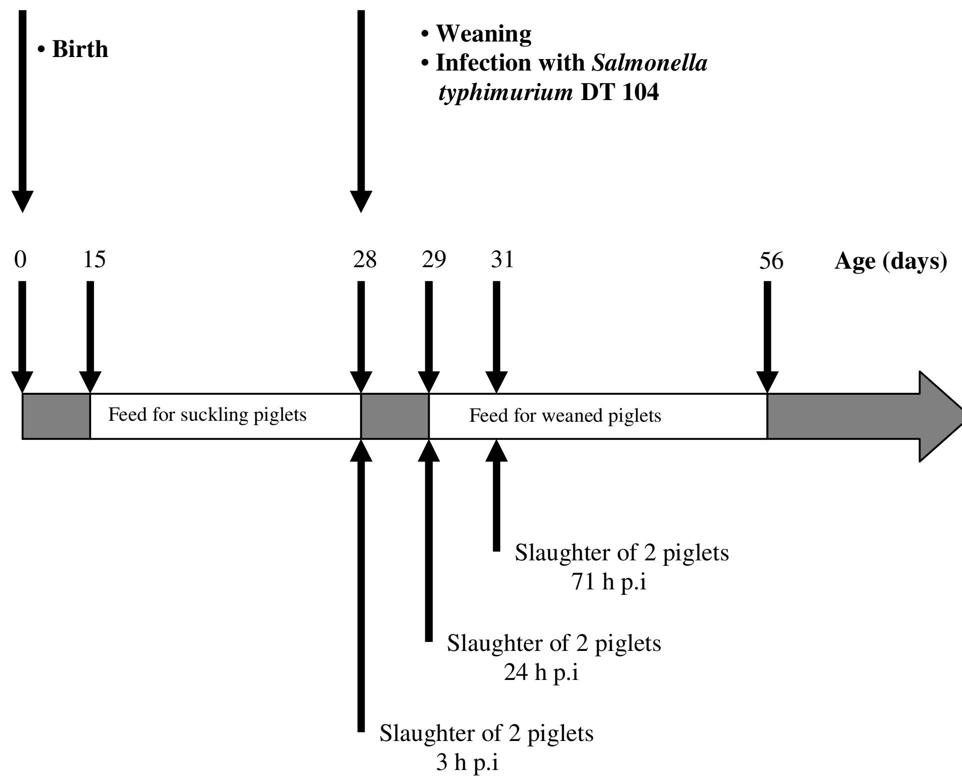

Fig. 4. *Schematic representation of the experimental design*

Table 1. Age groups of piglets sacrificed

Age of piglets	Time of sacrifice (h) p.i.	Number of piglets sacrificed from the probiotic-group	Number of piglets sacrificed from the control-group	Total number of piglets
28d	3	10	10	20
29d	24	10	10	20
31d	71	10	10	20
56d	672	10	8	18
Total number of piglets sacrificed				78

2.2.4. Collection of intestinal sections, spleen and blood

Samples used in this study are PBMC, discrete and distal continuous PP, spleen and IEL from the jejunum. Piglets were first anaesthetized with ketamin (0.2 ml/Kg) (10% Ketamin, Heinrich Fromme, GmbH) and azaperon (0.1 ml/Kg) (Stresnil von Janssen-Cilag) in a ratio of 2:1 and blood was taken from the *vena jugularis* and collected in a citrated tube. Subsequently, the piglets were euthanized by injection of pentobarbital (0.8 ml/Kg) (Eutha 77 von Essex) and the intestinal tract was removed and collected in PBS. Beginning with the end of the plica duodenocolica, 200cm of the proximal (discrete) jejunum were taken and transferred into PBS. Furthermore, 2 x 30 cm of the distal PP was taken and also transferred into PBS. Both intestinal samples were opened lengthwise and washed twice with PBS to remove ingesta. Fifty centimetre of the proximal jejunum was then transferred into collection medium (Hanks balanced salt solution without Ca2+ and Mg 2+, gentamicin 50 mg/100ml of HBSS, penicillin-streptomycin 2000 IU/2mg in 100ml of HBSS). Beginning from the proximal end of the jejunum, 3 x 2 x 0.5cm was cut to collect the discrete PP samples. The discrete and continuous PP and the jejunal (for later preparation of IEL) samples were transferred into separate vials with collection medium until processing.

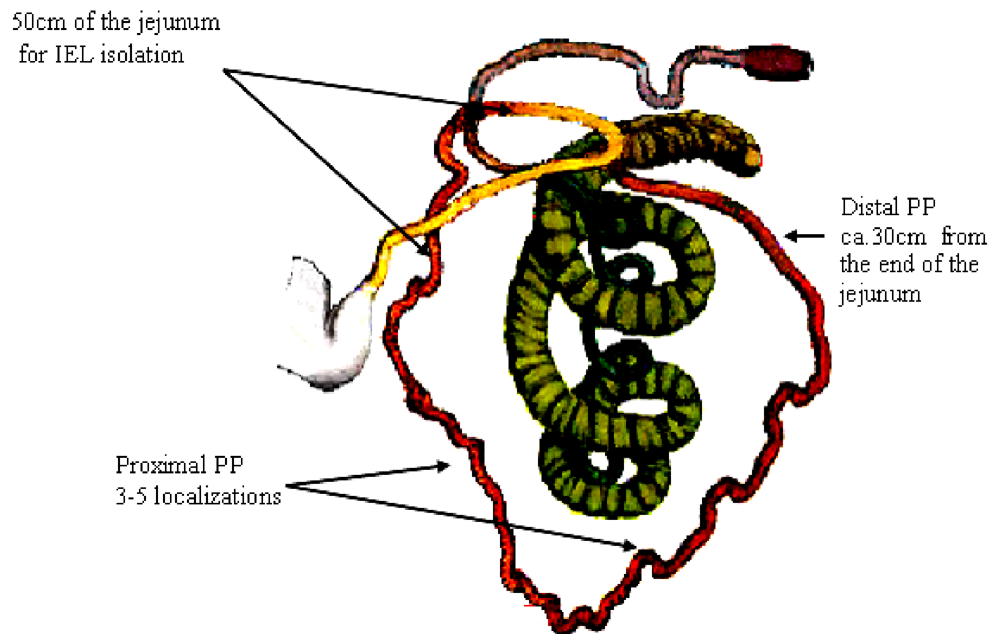

Fig. 5. *Collection of samples from the piglets´ intestine (Nickel et al., 1987).*

After collection of samples, the animals were killed by an overdose injection of pentobarbital and samples from the spleen of piglets of both groups were collected, put in transport medium and carried to the lab at 0°C. At 3h *p.i.*, PBMC, and spleen were collected. At 24h *p.i.*, discrete and distal continuous PP and IEL from the jejunum were collected. At 71h *p.i.*, distal continuous PP, spleen, IEL and PBMC were collected and finally, at 28d *p.i.*, spleen, discrete and distal continuous PP, IEL and PBMC were collected. The isolation of immune cells from blood, from discrete PP and distal continuous PP was carried out, unless otherwise indicated, at room temperature under a sterile condition according to the method described by Scharek et al. (2007). IEL isolation followed the method described by Solano-Aguilar et al. (2000) with some modifications. Below are the details of the steps followed during isolation of cells from the different tissues.

2.2.5. Isolation of lymphocytes from spleen, discrete and distal continuous PP

The isolated spleen samples were transferred to a petri dish with 3ml RPMI 1640 and cut into small segments (1-2mm^3) with scalpel blades. Together with the medium, the diced spleen suspensions were passaged through a 210µm Nylon mesh. Cells were washed once with RPMI 1640 and lysis of red blood cells was performed for 10min at RT using 5-10 volumes of lysis buffer (155mM NH_4Cl; 10mM $KHCO_3$; 0.1mM EDTA) for 1 volume cell suspension. The suspension was centrifuged at 320 x g for 10min and pellet was immediately suspended in 10ml RPMI 1640.

The discrete and distal continuous PPs were incubated for 20min in ca. 40ml Hank´s Buffered Salt Solution (HBSS) containing DTT at 37°C to remove the mucus. The tissues were cut on a petri dish with 3ml RPMI 1640 and suspensions containing the cells were passed through a 210µm nylon mesh in to a sterile beaker. This was repeated three times and the suspensions from each step were finally collected in a tube. About 20µl of DNase was added for a 50ml cell suspension, incubated for 5min at room temperature and centrifuged at 560 x g for 10min at room temperature. Pellet was resuspended in 15ml of 40% percoll in HBSS and 10-15ml of the suspension was carefully pipetted into tubes containing 5ml of 70% percoll. Centrifugation (1000 x g for 30min at room temperature) of the suspension resulted in three layers of cells: upper most layer containing dead cells, middle layer containing lymphocytes and lower layer containing erythrocytes. After removing the dead cells carefully, lymphocytes were collected in another tube and washed twice with ca. 5ml RPMI and centrifuged at 1000 x g for 30min at room temperature. To lyse possibly present erythrocytes, 5ml of lysis buffer

was added, incubated for 5min at room temperature and centrifuged at 300 x g for 10min at room temperature. Cells were resuspended in RPMI and counted while controlling the viability of the cells by 0.4% trypan blue (Sigma-Aldrich, St. Louis, USA) exclusion.

2.2.6. Isolation of intraepithelial lymphocytes from jejunum

For isolation of IEL, tissue sections taken from the proximal jejunum were cut into 3cm sections and incubated in HBSS with DTT (HBSS-DTT; HBSS without Ca2+ and Mg2+, 2mM DTT, 0.01mM Hepes) and gently shaken at 37°C for 5min. To release epithelial cells the medium was discarded and replaced with HBSS-EDTA (HBSS-EDTA; HBSS without Ca2+ and Mg2+, 1mM EDTA, 1mM Hepes) and further incubated for 35min at 37°C with gentle shaking. Suspensions were collected by passing through a sterile 210µm nylon mesh into a sterile beaker and the incubation step was repeated twice. The suspensions after each step were centrifuged at 600 x g for 10min at 4°C and cell pellets were resuspended in RPMI medium and kept on ice. All three suspensions obtained were combined and centrifuged. After resuspending the pellets in ca. 50ml RPMI, DNase treatment was performed as described above. Sediments were resuspended in 25% percoll in HBSS and centrifuged at 600 x g for 30min. The cell debris (top layer) was aspirated. The percoll solution was removed and the cell sediment was resuspended in 20-25ml RPMI. The suspensions were centrifuged at 320 x g for 10min at 4°C and pellets were resuspended in ca. 30ml PBS. The total viable cells in the suspensions were counted and used for further analysis.

2.2.7. Isolation of PBMC from blood

The mononuclear cell fraction of peripheral blood (PBMCs) of sows was isolated from citrated venous blood by density gradient sedimentation using Ficoll-Hypaque (Pharmacia Biotech Products, München, Germany). The blood samples collected were diluted 1 to 3 in PBS. Carefully, 30ml of the diluted blood was overlaid at the top of 12ml Ficoll-Hypaque (Pharmacia Biotech Products, München, Germany) solution. Centrifugation at 800 x g for 20min at room temperature resulted in different layers, from which the white layer (ca. 10ml) containing the PBMC was carefully collected into a sterile tube. Tubes were filled to 14ml with PBS and centrifuged at 320 x g for 10min at room temperature. Pellets were resuspended in 2ml PBS and 6ml lysis buffer was added and incubated for 5min at room temperature. Each tube was filled with PBS to 14ml and centrifuged (320 x g) for 10min at room temperature. Cells were resuspended in 1ml RPMI 1640 (Biochrom KG, Berlin, Germany), supplemented

with 100U/ml penicillin, 100mg/ml streptomycin, 2mM glutamine and 5% FCS (Biochrom KG, Berlin, Germany) and the total number of viable cells was counted.

2.2.8. Flow cytometry

2.2.8.1. Principle and application

A flow cytometer is an instrument that measures and analyses multiple physical characteristics of cells or other particles as they flow in a fluid stream through a beam of light. The illuminated cells display several types of signals which are detected, converted to digital data and further analysed by a computer. A flow cytometer that is equipped to sort the identified cells is called a Fluorescence Activated Cell Sorter (FACS). The technology relies on the fluidics, optics and electronics that enable simultaneous analysis of multiple characteristics of single cells at a very high rate.

A schematic picture of a flow cytometer is shown in Figure 6. A cell suspension is subjected through a nozzle whereby the cells are focused into the center of a columnar flow. The cells in the single cell stream are exposed to a light source from a laser beam. The cell cytometer used in this study has an argon laser beam that emits light at a wave length of 488nm. Each cell scatters the laser light, which gives information about the size (forward scatter light, FSC) and granularity of the cell (side scatter light, SSC) (Fig. 7).

Principle of flow cytometry

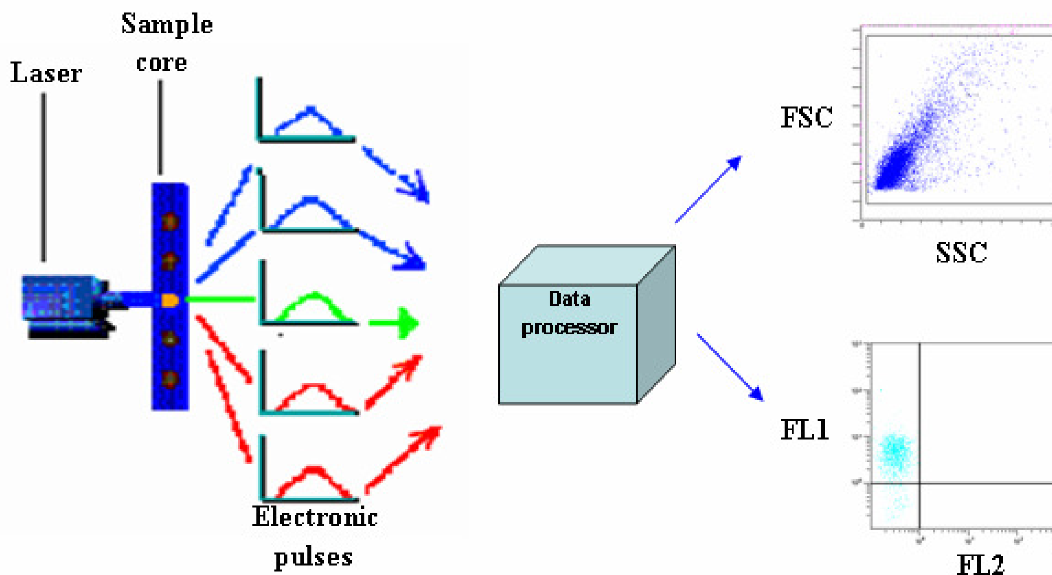

Fig. 6. *A flow cytometer system has three major components: The fluidics system that transports the cells in a stream, the optics system that consists of lasers to illuminate the cells in the sample stream; and the electronics system that converts the detected light signals into electronic signals that can be processed by the computer. FL implies to fluorochrome and each dot in the dot-plot represents one cell.*

Light scatter versus cell properties

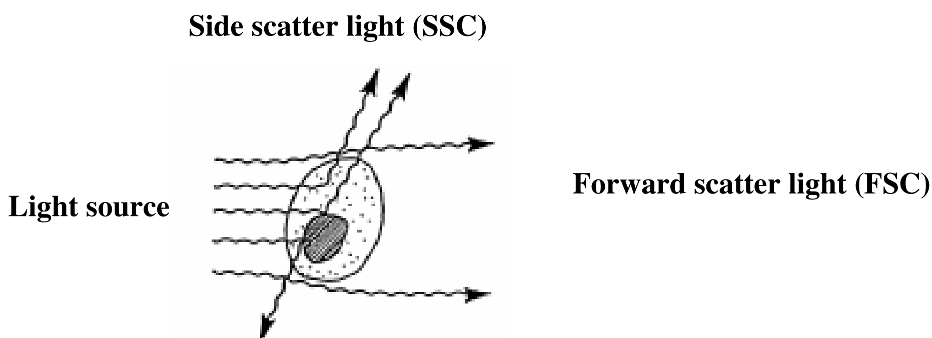

Fig. 7. *A cell interrogated with a light source emits forward scatter light (FSC), which is proportional to the cell size and side scatter light (SSC), which is proportional to the granularity of the cell.*

2.2.8.2. Flow cytometric staining and phenotyping

The percentage of CD4+ cells in the freshly isolated lymphocytes from discrete PP, spleen and that of CD8+ cells in the IEL was determined using a 488nm argon laser equipped Coulter®EPICS® XL-MCL-flow cytometer (Beckman Coulter) that was calibrated with Flow-Check®-Fluorospheres (Beckman Coulter). The fluorochromes used for the analysis were fluorescein isothiocyanate (FITC), phycoerythrin (PE) and propidium iodide (PI). FITC has an absorption spectrum of 495nm and an emission spectrum of 519nm; PE has an absorption spectrum of 480-565nm and an emission spectrum of 580nm and PI has an absorption spectrum of 550nm and an emission spectrum of 580nm.

Briefly, 1.0 x 10^7 cells were washed twice with PBS and resuspended in 90µl of PBS-0.5% BSA-2mM EDTA solution. To determine the percentage of CD4+ cells, 10µl of mouse anti-porcine CD4a conjugated to FITC (clone 74-12-4, Biozol Diagnostika D-85386) was added. In the case of the IEL samples, 10µl of mouse anti-porcine CD8a, conjugated to R-PE (clone PT8, Biozol Diagnostika) was added and incubated for 20min at 4°C (Fig 9). Cells were washed with PBS-0.5% BSA-2mM EDTA solution and centrifuged at 300 x g for 10min. The pellet was resuspended in 80µl PBS-0.5% BSA-2mM EDTA solution and 20µl of magnet cell sorting (MACS) goat anti-mouse IgG micro bead (Miltenyi Biotec GmbH) was added for further isolation of pure cell populations. Cells were incubated for 20min at 4°C, washed twice and resuspended in 3ml buffer of which 200µl was taken for FACS analysis and the rest was used for MACS (Fig. 9 and Fig. 10). The 200µl volume taken from each sample was mixed with 1ml PBS-0.2% BSA solution and analysed immediately on a flow cytometry after adding PI (0.5 µg/ml) (Sigma-Aldrich) to each sample. Analysis of flow cytometric data was completed using Expo®32 MultiCOMP-Software (Beckman Coulter). The gated region represents the percentage of CD4+ (X-axis) or CD8+ cells (Y-axis), from which 10, 000 events were collected at a flow rate of 100µl/min (Fig. 8). On a dot-plot made SSC versus FSC, dead cells that are stained with PI were excluded from further analysis. Using a quadrate marker, the dot plot made FL1 (FITC) versus FL2 (PE) divided the two parameter plots into 4 sections (Fig. 8). The bottom left area shows cells that bound neither antibodies conjugated with FITC nor PE; CD4-CD8-, the right bottom part shows cells that are bound to FITC- and not to PE-conjugated antibody, CD4+CD8-, cells that are bound to PE- and not to FITC-conjugated antibody, CD4-CD8+, are shown on the upper left area while cells that bound antibodies conjugated to either FITC or PE, CD4+CD8+ cells, are represented in the upper right side of the graph (Fig. 8).

Flow cytometry data presentation

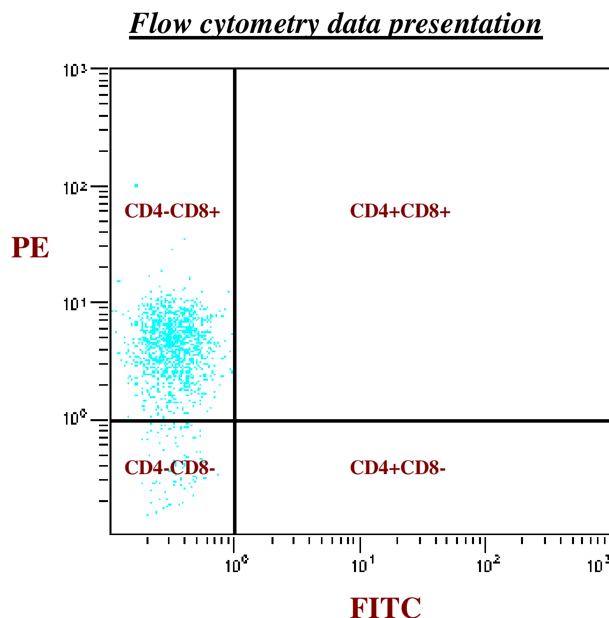

Fig. 8. *A dot-plot presentation of a flow cytometric analysis showing the areas of the plot that represent negative, single positive and double-positive cells for CD4 and CD8 phenotypes*.

2.2.9. Magnetic activated cell sorting (MACS)

MACS® Column Technology is based on the use of MACS MicroBeads, MACS Columns and MACS Separators. Magnetic cell sorting (MS) columns have been developed for the gentle isolation of MicroBead labeled cells. As MACS MicroBeads are extremely small, a high gradient magnetic field is required to retain the labeled cells. MS columns contain an optimized matrix to generate this strong magnetic field when placed in a permanent magnet such as the MiniMACS™ Separator (Miltenyi Biotec GmbH).

The separation of CD4+ cells from lymphocytes of the discrete PP and spleen, and CD8+ cells from lymphocytes of the IEL was done using MS columns (Miltenyi Biotec GmbH) inserted in the magnetic field of MiniMACS™ Separator (Miltenyi Biotec GmbH) according to the manufacturer's instructions. The hydrophobic coating of the columns was washed out with 1ml PBS buffer (PBS with 0.5%-BSA and 2mM EDTA). About 3ml of the magnetically labelled cells after immuno-staining with the respective antibodies described above, were separately passed through the column. The MS column was washed twice with 1ml degassed PBS buffer at a time. Unlabeled cells that passed through the column were collected in a tube and discarded. The MS column was removed from the magnetic field and placed on a new collection tube. The positive fraction of the cells was collected by adding 1ml PBS buffer and

firmly applying the plunger supplied with the column (Fig. 10). This step was repeated in order to collect as much positive cells as possible. Viable cells after isolation were counted and the cell pellets were resuspended in ca. 300µl PrepProtect™ stabilization buffer (Miltenyi Biotec GmbH) in order to prevent RNA degradation during storage at -80°C.

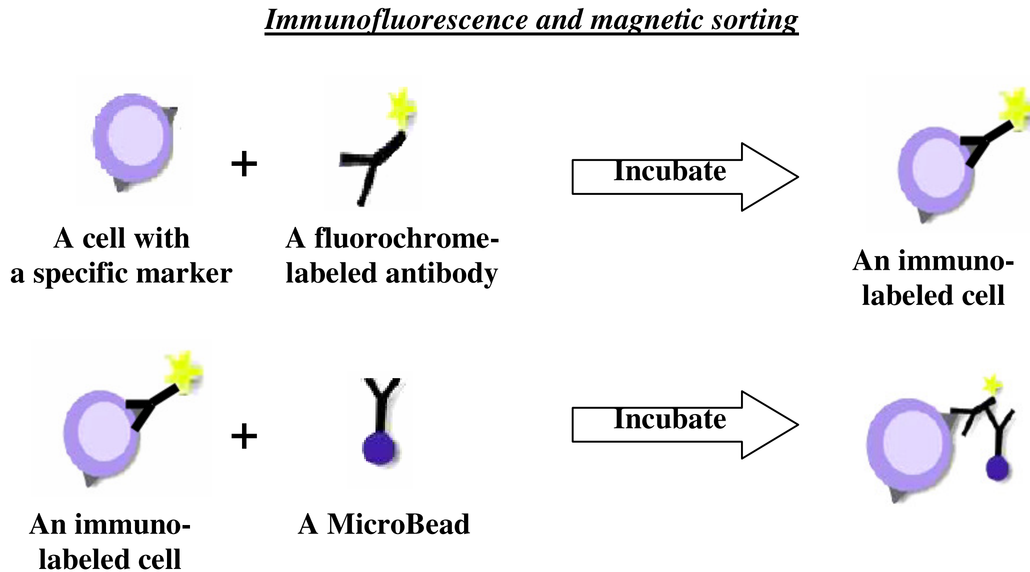

Fig. 9. *Incubation of cells with fluorochrome bound primary antibodies enabled the phenotypic analysis of the lymphocyte population isolated from the different organs. Incubation of the immuno-labeled cells with MicroBeads was required for the separation of specific lymphocyte populations by magnetic cell sorting (MACS) method.*

Magnetic cell sorting

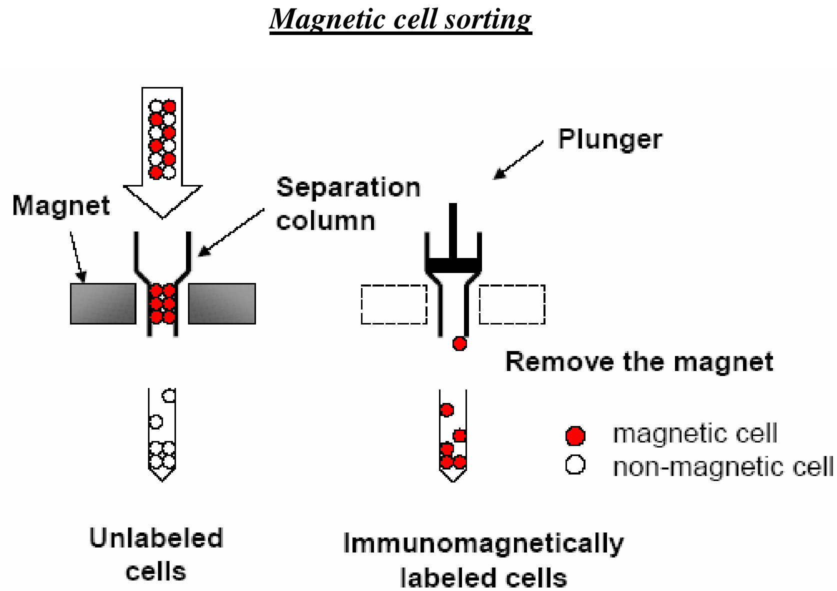

Fig. 10. *After incubation with MicroBeads, cell suspension is loaded on to an MS column. The unlabeled negative cells run through while the immunomagnetically labeled positive cells are retained on the MS column. Negative cells are discarded and positive cells are collected in a separate collection tube after removing the column from the magnetic field.*

2.2.10. Relative quantification of selected genes using real-time PCR

2.2.10.1. Principles of real-time PCR

Quantitative PCR is a PCR, where the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. In contrast to end point quantitative PCR, in real-time PCR fluorescent signal is measured during the amplification of the product. This allows quantification of the template to be based on the fluorescent signal during the exponential phase of the amplification, before limiting reagents, accumulation of inhibitors, or inactivation of the polymerase have started to have an effect on the efficiency of the amplification (Stratagen, 2004). The fluorescence intensity increases proportionally to the concentration of the amplicon. The first cycle at which the real-time PCR machine can distinguish the amplification generated fluorescence as being above the ambient background signal is called the threshold cycle or Ct. The Ct value is inversely proportional to the initial copy number of the gene. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample (Fig. 11).

Principles of real-time PCR

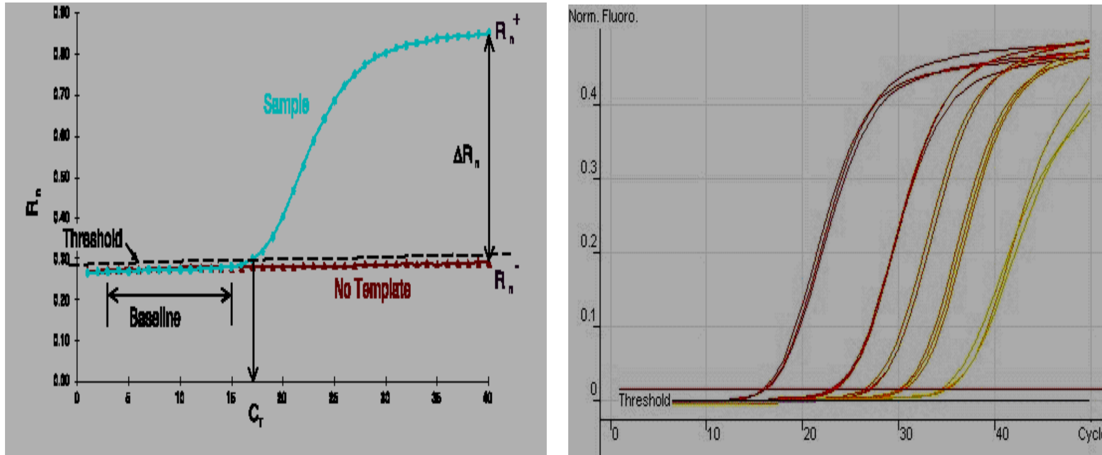

Fig. 11. *The Ct values are inversely proportional to the initial copy numbers. Thus, a higher initial copy number will correlate to a smaller Ct value.*

2.2.10.2. RNA isolation and cDNA synthesis

A total of $1x10^7$ cells isolated from blood (PBMC) and continuous PP and stored at -80°C in PrepProtect™ stabilization buffer (Miltenyi Biotec GmbH) were centrifuged at 1500 rpm for 5min and the medium was completely removed. Cell pellets were loosened by flicking and total RNA was isolated using Invisorb® Spin Cell RNA Mini Kit for total RNA extraction from human and animal cell culture (Invitek, Germany) according to the supplied protocol. Then 0.5µg of total RNA was subjected to DNase degradation in a total volume of 20µl containing DNase, buffer and water to eliminate residual genomic DNA. The mixture was incubated at 25°C for 10min, at 42°C for 1hr and finally at 70°C for 10min. The DNase-treated RNA samples were reverse-transcribed to synthesize cDNA by the enzyme Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase according to the manufacturer's instructions (Invitrogen, Germany). Reverse transcription took place in mastercycler gradient with incubation at 25°C for 10min, 42°C for 1hr and 90°C for 2min and was cooled to 4°C. The quality and concentration of samples were determined using nanodrop spectrophotometer ND-1000 (PEQLAB Biotechnology, Germany) by measuring optical density at 260nm.

2.2.10.3. Primer design

Primers designed by Dr. Christoph Gabler (Department of Biochemistry, Faculty of Veterinary Medicine, FU) using the Husar sequence analysis package were used. The specificity of the genes amplified using the primers was confirmed by performing BLAST searches. All primers were synthesized by TIB-Molbiol (Berlin, Germany). Primers were dissolved in PCR-H_2O to a concentration of 200µM and kept as a stock solution at -20°C until use. For each PCR reaction, 20µM primers were prepared from the stock solution by further diluting with PCR-H_2O. Primer optimization was performed before using the primers for real-time PCR. The description of the primers used is shown in Table 2.

Table 2. Primers used for the *in-vivo* study

Gene	F/R	Sequence (5´-3´)	Annealing temp.	Product	Accession No.
IL-1ß	F R	CAAGGAGATGACAGCGATGA GGGCTTTTGTTCTGCTTG AG	59°C	252bp	AY577818
IL-1α	F R	AGGGTCATCAACCACCAGTG GTTTTGGGTGTCTCAGGCAG	58.5°C	257bp	NM_214029
IL-2	F R	CTAACCCTTGCACTCATGGC ATTCTGTAGCCTGCTTGGGC	60°C	184bp	EU139160
IL-6	F R	CAGCAAGAAGGTACTGGCAG CTCAGGGTCTGGATCAGTGC	58.5°C	225bp	NM_214399
IL-8	F R	AACTGGCTGTTGCCTTCTTG ACCTTCTGCACCCACTTTTC	58°C	253bp	NM_213867
IL-10	F R	TAAGGGTTACCTGGGTTGCC TGGGGGTTACCTGGGTTGCC	58°C	254bp	NM_214041
IFN-γ	F R	CCAGGCGCCCTTTTTTAAAG AGTCTCCTTTGAATGGCCTG	59°C	210bp	NM_213948
TNF-α	F R	TCAAACCTCAGATAAGCCCG AGTGAGGAAAACGTTGGTGG	59°C	255bp	X57321
CD9	F R	ACTATGCGCTGGACTGCTG GATGGCACAGCACAGGATC	63°C	224bp	NM_214006.1
TLR2	F R	ACATGAAGATGATGTGGGCC TAGGAGTCCTGCTCACTGTA	59°C	107bp	AB208696
TLR9	F R	CGCACAATGACATCCATAGC AAGGCCAGGTTATTGTCACG	59°C	253bp	NM_213958
TGF-ß	F R	CTCTCTCGGCAGAGCTG AGTTGGTATCCAGGGCTCG	60°C	403bp	AF461808
IL-1ra	F R	TTGAGCCTCATTTTGTGTTCC GTGTTGGTGAGGCCAACAG	60°C	256bp	NM_214262
Cox 1	F R	CAAAGGGAAGAAGCAGTTGC AATGTGGCCGACGTCTACC	63°C	205bp	AF207823
Cox 2	F R	ATGATCTACCCGCCTCACAC GCAGCTCTGGGTCAAACTTC	60°C	280bp	AF207824
iNOS	F R	ATGTTCGAGCACATCTGCAG GTATTTGGGGTGTTCCATGG	60°C	342bp	U59390
18S-rRNA	F R	AATCGGTAGTAGCGACGGG AGAGGGACAAGTGGCGTT	59°C	276bp	AY265350

2.2.10.4. Reverse transcription polymerase chain reaction (RT-PCR)

Gradient PCR was carried out in Mastercycler gradient using the above set of primers using 18s rRNA gene as a house keeping gene. For primer optimization, 12 annealing temperatures were tested for each primers in the range of 60 ± 8°C. A PCR reaction contained 2.5µl of 10x iTaq buffer, 0.75µl of $MgCl_2$ (50 mM), 0.5µl of the four dNTPs (each 10mM), 0.5µl of each sequence specific primer (20µM), 17.25µl of PCR-H_2O and 0.1µl of iTaq-Polymerase (5U/µl). The cycling conditions were: One cycle of 94°C for 2min; 45 cycles of denaturation at 95°C for 15sec; annealing at 60 ± 8°C for 20sec; extension at 72°C for 30sec, followed by final single extension cycle at 72°C for 10min. PCR products were separated by electrophoresis (70volts for 60min) on a 2% Tris-acetate-EDTA (TAE) agarose gel (200ml) with 0.5µg ml-1 ethidium bromide and their sizes were determined by comparing with DNA molecular weight markers run together. The PCR bands were visualized using UV transilluminator and temperatures at which strongest bands were obtained were selected as the optimal annealing temperatures of the primers (Table 2) to be used in real-time PCR.

2.2.10.5. Real-time PCR measurement and analysis

The expression profile of the above genes was studied by real-time-PCR using a 10µl reaction volume containing 0.2µl forward primer (20µM), 0.2µl of reverse primer (20µM), 0.2µl of 50x SYBR Green Solution, 3.4µl PCR-H_2O, 5µl 2x SensiMix (dT) with either 1µl cDNA sample or for a negative control 1µl PCR-H2O or 1µl of standard. Reaction tubes were put in a Rotor Gene RG-3000 and amplification was done using the program indicated in Table 3.

Table 3. PCR amplification conditions

PCR Program		Temperature	Time	Cycle number
Activation of DNA polymerase		95°C	10min	1
Cycling	Denaturation	95°C	15sec	45
	Annealing	Variable (Table 1)	20sec	
	Extension	72°C	30sec	
Melting of the amplicon		50-99°C	5sec/1°C	1

Purified PCR products served as external quantification of the respective cDNA. They were 10-fold serially diluted (1:10^3-1:10^9). Instead of cDNA, PCR-H_2O or samples with out reverse transcriptase enzyme served as negative controls. The 18s rRNA gene was used as endogenous reference for normalization of Ct values of target genes. Each sample was

analyzed in triplicate. Analysis of the real-time PCR results was done using the software Rotor-Gene 6.0. The melting curves of the products were used to confirm the specificity of the reactions. A standard curve using the logarithmic values of the known standard concentration and their Ct-values was calculated, from which the initial expression amount of each gene could be determined by the software.

2.3. *In-vitro* evaluation of the effect of *E. faecium* SF68 against TGEV

2.3.1. Isolation of cells

Cells previously isolated from the intestine two different 56 days old piglets were used for this part of the study. The isolation targeted epithelial cells following a modified method for isolation of lymphocytes from swine intestine described by Solano-Aguilar (2000). Briefly, tissue from jejunum were cut in 3cm pieces, mixed with 30-50ml HBSS-DTT-Medium and incubated at 37°C for 5min. The suspensions were filtered and mixed with HBSS-EDTA and incubated at 37°C for 30min. The suspensions were filtered and supernatants were stored at 4°C until the following step. After 3 repeated incubations, tissues were discarded and all supernatants collected at each step were centrifuged at 600 x g, 4°C for10min. Each pellet was resuspended in 10-15ml RPMI and the three supernatants were missed in a single tube to which 50 µl DNase (5mg/ml) was added followed by incubation at 37°C for 5min and centrifugation at 600 x g for 10min. The pellets were resuspended in 25% percoll and centrifuged at 600xg, 20min at room temperature (RT). Pellets were resuspended in 5-10 volume lysis buffer and incubated at RT for 5min. The suspension was centrifuge at 300 x g, 10min and the pellet was resuspended in 35ml RPMI. Cells were directly seeded into 25cm^2 culture bottle containing Dulbecco’s modified Eagle’s medium (DMEM) (PAN Biotech) supplemented with 10% fetal calf serum (FCS) (Hyclone) and 1% penicillin/streptomycin (Biochrom) and were referred to as type II cells. In the case of type I cells, the suspension in RPMI was pelleted, resuspended in 40% percoll and centrifuged at 600 x g for 20min. The pellet was resuspended in DMEM (supplemented with 10% FCS, 1% Penicillin/streptomycin) and centrifuged at 600 x g for 5min. The pellet was resuspended in DMEM and was cultured in another 25cm^2 culture bottle containing DMEM (10% FCS, 1% Penicillin/streptomycin).

2.3.2. Growth and cell- size characterization of Type I and Type II cells

The morphology of the cells was analyzed visually by light microscopy of confluent cultures. The growth curve, cell size and granularity of the cells were studied by growing the cells in DMEM supplemented with 10% FCS and 1% Penicillin/Streptomycin. Incubation was carried out at 37°C in a 5% CO_2 atmosphere. The cells were monitored on a daily basis to record their phenotype and were photographed under a phase-contrast microscope. To determine the growth curves of the cells, type I cells and type II cells were separately seeded into 24-well plates at a density of 5 x 10^3 cells/well and 1.5 x 10^4 cells/well, respectively. After incubation, the cells were trypsinized and counted using a hemocytometer every day. All experiments were performed three times and results were presented as the average.

Flow cytometric analyses of type I and type II cells were done to estimate the comparative size and granularity of the cells. Cells were harvested after trypsinization and a total of $2x10^5$ cells were suspended in 1ml of PBS-0.2% BSA solution. FACS measurements were done after adding 1µl of PI in the cell suspensions. The morphological profile of the cells was analysed on a dot-plot made of forward scatter light (FSC) versus side scatter light (SSC), excluding dead cells that are stained with PI. The FSC measurement was related to the cell size while SSC was associated with the internal granularity of the cells.

2.3.2.1. Growth of type I and type II cells in three-dimensional (3D) culture

Cells were grown in culture flasks and trypsinized on confluency. Cells were washed and pelleted by centrifugation at 200 x g for 5min. Cells were encapsulated in alginate microspheres according to the previously described protocol (Madry *et al.,* 2003). Briefly, cells were suspended in filter sterilized 1.2% alginate in 0.15M NaCl at $5x10^5$ cells ml-1. The cell suspensions were extruded through a 21-gauge needle into a 102mM CaCl2 solution at room temperature (RT) under constant shaking and allowed to polymerize for 10min. The resulting implants were washed twice in 0.15M NaCl followed by two consecutive washes in basal medium and placed in growth medium. Alginate microspheres were incubated in growth medium at 37°C in a 5% CO_2 atmosphere and the growth of the cells was controlled under a microscope. Entrapped cells were released from alginate beads after two weeks. Individual microspheres were solubilized by incubating them in 100µl 55mM sodium citrate with 90mM NaCl (pH 6.8) for 20min at RT. The released cells were counted and their viability was determined by trypan blue staining.

2.3.3. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) of trypsinized preparations of type I and type II cells in 1.5ml eppendorf tubes was performed for the ultra-structural characterization of type I and type II cells. Additionally, non-trypsinized preparations of both cell types were examined under TEM in order to know the presence of cell-to-cell contact in monolayer cultures.

Cells were washed once with PBS (0.01M, pH 7.4) and finally with 0.1M cacodylate buffer (pH 7.2). Cells were fixed with Karnovsky-fixative (2% paraformaldehyde und 2.5% glutaraldehyde in 0.1M cacodylate buffer, Merck Eurolab GmbH, Darmstadt) at 4°C. After washing three times with 0.1M cacodylate buffer, cells were contrasted with 1% osmiumtetroxide and 1.5% potassium ferrocyanide (in 0.1M cacodylate buffer) in darkness for 2hrs at 4°C. Cells were washed three times with 0.1M cacodylate buffer and centrifuged at 200 x g for 5min. The cell pellets were resuspended in 1.5% Agar (in Aqua dest.) and incubated at 4°C for 30min. Finally the agar containing the cell pellet is carefully taken out of the tube, cut into smaller pieces and washed with 0.1M Cacodylate buffer. The pellet was dehydrated in a series of graded ethanol: 50%, 70%, 80%, 90% (each 15 min) and 100% (twice 15min) and in 100% propylene oxide (Serva Electrophoresis GmbH, Heidelberg) for 15min, and finally in an equal volume of 100% propylene oxide and epoxy resin for about two hours. Samples were transferred to Beem capsules (PLANO, Marburg, Germany) and were polymerized in pure epoxy resin (Epon) for 4hrs. Samples were further incubated in epon at 60°C for 48hrs. Epon blocks were semi-thin sectioned (1µm thick) with ultramicrotome (Reichert-Jung, Wien, Austria), stained with methylene blue (Sigma-Aldrich Chemie GmbH, Deisenhofen) and examined under light microscopy to get orientation for the ultra-thin sections preparation. Finally ultra-thin sections (60nm) were prepared from the appropriate samples, mounted on 400-mesh copper grids (PLANO, Marburg, Germany) and post-stained with lead acetate (Leica Microsystems AG, Wetzlar) and lead citrate (Leica Microsystems AG, Wetzlar) each for 10min. The grids were examined under a transmission electron microscope 10 CR (Carl-Zeiss, Jena) and pictures were documented by Kodak electron microscope film (Kodak GmbH, Stuttgart).

2.3.4. Flow cytometric and histochemical analysis of expression of cell markers

2.3.4.1. FACS analysis

FACS analysis was undertaken to identify type I and type II cells using primary antibodies for endothelial cells markers, the von Willebrand factor (VWF) glycoprotein (1:200) and CD31 (PECAM-1), (1:200) for the leucocytes marker, CD45 (1:200) and the macrophage and monocytes marker, CD14 (1:200). About $2x10^5$ cells were washes with PBS and resuspended in 98µl PBS-0.2% BSA solution and 2µl of the primary antibody was added and incubated at 4^oc for 20min. Cells were washed with PBS-0.2% BSA solution and centrifuged at 300 x g for 10min. The pellet was resuspended in 98µl PBS-0.2% BSA solution and was incubated with 2µl of secondary antibody conjugated with FITC at 4^oC for 20min. Cells were washed with 3ml of PBS-0.2% BSA solution and centrifuge at 300 x g for 10min. Cells incubated only in PBS were used as negative control to detect non-specific reactions. Pellets were resuspended in 100µl PBS-0.2% BSA solution and 1µl of PI was added. FACS analysis was done using a 488nm argon laser equipped Coulter® EPICS® XL-MCL-flow cytometer (Beckman Coulter) that was calibrated with Flow-Check®-Fluorospheres (Beckman Coulter).

2.3.4.2. Confocal fluorescent microscopy

Type I and type II cells were further characterized histochemically for the expression of cytokeratin and vimentin. Pan-cytokeratin serves as a marker for epithelial cells (Blair et al., 1995; Schierack et al., 2006) while vimentin is a mesenchymal marker expressed by fibroblasts, endothelial cells, plasma cells, lymphocytes and perivascular macrophages but not much expressed by epithelial cells (Kokkinos, et al 2007; Koshi et al., 2001). Cells ($1x10^5$) were grown on coverslips inserted in a 24-wells plate and fixed with acetone for 5min at -20^oC. The coverslips were washed with PBS (2x) and incubated with 50µl of the primary antibodies pan-cytokeratin (clones A1 and A3, dilution 1:200 in PBS-5% BSA; Serotec, Germany), which stains multiple cytokeratins of high (1, 2, 3, 4, 5, 6, 10, 14, 15 and 16) and low molecular weight (7, 8 and 19), or vimetin (1:400 in PBS-5%BSA) in a separate well for 90min at 4^oC. Cells were washed with PBS (2x) and incubated with a secondary antibody (FITC-conjugated goat antimouse antibodies diluted 1:100; Beckman Coulter, USA) for 90min at 4^oC protected from light. Immunofluorescence microscopy was performed with a Leica TCS SP2 confocal laser-scanning microscope (Leica, Germany) and images were processes using Adobe Photoshop.

2.3.5. Analysis of gene expression

Total RNA was isolated from both type I and type II cells using TRIsure reagent (Bioline, Germany) according to supplied protocol. To remove genomic DNA contamination, DNA digestion was performed before reverse transcription (Huang *et al.* 1996). DNase treatment was carried out in a total volume of 10µl containing 1µg total RNA, 1µl DNase (Invitrogen) and the 1µl 10x DNase buffer. DNase was deactivated by adding 25mM EDTA and incubating at room temperature for 15min followed by heating the mixture at 65°C for 10min. DNase treated RNA samples were reverse transcribed to cDNA using MMLV reverse transcriptase according to the manufacturer's instructions (Invitrogen, Germany). The quality and concentration of samples were determined using nanodrop system by measuring optical density at 260nm.

2.3.5.1. Reverse transcription polymerase chain reaction (RT-PCR)

The expression of selected cytokines (IL1ß, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α) and mucin 2 in type I and type II cells was studied by RT-PCR using ß-actin as a house-keeping gene. The sequences of the primers, the sizes of the products and the specific annealing temperatures are shown on Table 4. The primers for IL-6, IL-8 and IFN-γ were designed from the corresponding sequences from GenBank using the program Primer Premier 5.0. The other primers were published. All primers were synthesized by TIB-Molbiol (Berlin, Germany).

Two microgram cDNA was amplified in a total reaction volume of 25ml containing 1x buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.4pm of each primer (forward and reverse) and 0.0148 U of Taq DNA polymerase. Each reaction started with an initial denaturation step for 3min at 94°C followed by 40 cycles of the amplification program, 94°C for 45 sec; annealing 53-55°C for 45sec and extension at 72°C for 45sec. As a final step, an elongation phase was performed for 10min at 72°C before samples were cooled down to 4°C. Subsequently, 8µl PCR product were subjected to 2% tris-acetate-EDTA (TAE) agarose gel electrophoresis containing 0.5µg ml-1 ethidium bromide at 1-5volts/cm for 20-25min. Bands were visualized using UV transilluminator and digital photographs were documented.

Table 4. Primers used for *the in vitro* study

Gene	F/R	Sequence (5´-3´)	Annealing temp.	Product	Accession No.
IL-1ß	F	AAAGTTACCCGAAGAGGGACA	55°C	119bp	AY577818
	R	TGCTTGAGAGGTGCTGATGTA			
IL-2	F	GCCCAAGCAGGCTACAGAAT	60°C	73bp	EU139160
	R	TAGCACTCCCTCCAGAGCTT			
IL-4	F	TACCAGCAACTTCGTCCAC	60°C	150bp	NM_214123
	R	TCCTTCTCCGTCGTGTTCT			
IL-6	F	AACGCCTGGAAGAAGA	53°C	229bp	NM_214399
	R	AACCCAGATTGGAAGC			
IL-8	F	GTTCTGGCAAGAGTAAG	53°C	275bp	NM_213867
	R	CACGGAGAATGGGTTT			
IL-10	F	CATCAATTTCTGCCCTGTGA	60°C	71bp	NM_214041
	R	TGGAGCTTGCTAAAGGCACT			
IFN-γ	F	CTCCATCCTGGCTGTG	53°C	183bp	NM_213948
	R	CATTTCCGAGATGAACC			
TNF-α	F	GACCAGCCAGGAGAGAGACA	56°C	106bp	X57321
	R	GCGTGTGAGAGGGAGAGAGT			
ß-actin	F	GGACTTCGAGCAGGAGATGG	55°C	233bp	DQ845171
	R	GCACCGTGTTGGCGTAGAGG			

2.3.6. Determination of the sensitivity of type I and type II cells to TGEV infection

Type I and type II cells and the model cell line for studies on TGEV, epithelial McClurkin swine testis (ST) cell line were cultured in $60mm^2$ culture plates containing DMEM supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C in 5% CO_2 atmosphere. On confluency, monolayers were washed with PBS and infected with TGEV in DMEM (with out supplements) at an MOI (multiplicity of infection) of 10. The cultures were further incubated and cytopathic effect (CPE), which is characterized by morphological changes of the cells as a result of viral infection, such as formation of cell rounding, swelling or shrinking, death, detachment from the surface, etc., were observed in comparison to control cultures that were not infected and photographs were documented using a phase-contrast microscope.

Transmission electron microscopy (TEM) was used in-order to confirm the presence of the virus inside the cells. Twenty four hours after infection, monolayers of type I and type II cells were collected carefully into 1.5 ml eppendorf tubes and washed once with PBS (0.01M, pH 7.4) and 0.1M cacodylate buffer (pH 7.2). Cells were fixed with Karnovsky-fixative at 4^oC. The fixed cells were pre-treated for TEM as described above. Pictures were taken under a transmission electron microscope 10 CR (Carl-Zeiss**,** Jena) and documented by Kodak electron microscope film (Kodak GmbH, Stuttgart).

2.3.7. Evaluation of the effect of the probiotic against TGEV infection in type II cells

To perform biological assays, 3×10^4 type II cells were separately seeded per well in 96 well plates, as determined by trypan blue viability staining, and incubated for 24 h at 37°C in atmosphere of 5% CO_2 to reach the monolayer. Just before use, the monolayers were washed twice with 100µl DMEM without supplements.

The probiotic strain *E. faecium* SF68 was a generous gift from Prof. Dr. Ortwin Simon, managing director of the Institute of Animal Nutrition, Department of Veterinary Medicine, Freie Universität Berlin. The strain was grown in Brain Heart Infusion (BHI) Broth at 37°C and growth of the culture broth was monitored turbidometrically by measuring the optical density at 600nm (OD_{600nm}). The OD_{600nm} reading was converted to CFU/ml using the experimentally predetermined conversion factor for *E. faecium* SF68, which related the OD_{600nm} to CFU. Bacterial cultures were then centrifuged at 2400rpm for 10min and the pellet were washed twice to remove excess BHI broth and resuspended in DMEM (without supplements). The final bacterial suspension contained 1×10^8 bacterial cells ml−1, which was then 10 fold serially diluted in DMEM (without supplements) for further experiments. The culture supernatant was collected in a separate container and filter-sterilized after adjusting the pH value to 7.0 using NaOH (1mol/l). Serial two-fold dilution of the supernatant in DMEM (without supplements) was used for further analysis.

2.3.7.1. Virus

TGEV was passed several times in type II cells. Supernatant containing the virus was collected from infected monolayers of type II cells 48hrs after infection by freezing and thawing the culture plates repeatedly followed by clarification of the virus containing suspension by centrifugation at 2400rpm for 10min. Virus was stored at -80°C until used. For the antiviral assay using type II cells, virus with $10^{7.7}$ tissue culture infective dose 50% units per ml (TCID50 ml-1) was used after determining the virus titre on ST cells as described below. In all cases, 100µl of a particular dilution of the virus suspension was applied to a well of 96-wells micro-culture plates containing confluent monolayers of the cells.

2.3.7.2. $TCID_{50}$ determination

TGEV was titrated on 96-well plates containing confluent 24hrs-old ST cells. Virus supernatants were 10-fold serially diluted (10^0 to 10^{-8}) in DMEM (without supplements). Growth medium was discarded and monolayers were washed with 1% PBS. Eight wells were

inoculated with 100µl of virus suspension at each dilution. After incubating for 2hrs, the inoculum was discarded, monolayers were washed two times with PBS (1%) and 100µl of DMEM (supplemented with 1% FCS and 1% penicillin/streptomycin) was added to each well. Plates were incubated at 37°C in a humidified 5% CO_2 incubator for 48-72hrs. Virus titres ($TCID_{50}$ values) per ml of suspensions were calculated by the Reed–Muench method, which is based on the number of wells showing CPE (Krah, 1991). All experiments were done in triplicate. Unless otherwise mentioned, the cells used for viral titration were ST cells.

2.3.7.3. Methylthiazol-diphenyltetrazolium bromide (MTT) Test

MTT assay is used to measure mitochondrial function, which serves as an index of living cells. In this study, we used the assay in order to determine the effect of the probiotic on the infectivity of TGEV. The MTT substance is reduced by mitochondrial dehydrogenases in living cells to a blue-magenta colored formazan precipitate. The absorption of dissolved formazan in the visible region correlates with the number of intact alive cells. Viral infections cause cytotoxicity that damage and destroy cells and thus decrease the reduction of MTT to formazan (Mosmann, 1983).

The assay was carried out as previously described by Edmondson et al. (1988). Washed monolayers of type II cells (5 x 10^4 cells) on 96-wells plates were incubated with various dilutions (10^8, 10^7, 10^6 or 10^5) of viable probiotic bacteria or different dilutions (2^{-1}, 2^{-2}, 2^{-3}) of the probiotic culture supernatant (100µl/well) in DMEM (without supplements) for 2hrs at 37°C in the atmosphere of 5% CO_2. After incubation, 50µg/ml of gentamicin in DMEM was added to prevent bacterial overgrowth and secretion of bacterial metabolites and plates were further incubated for 1hr. The monolayers were washed (2x) with DMEM (without supplements) and challenged with $10^{7.7}$ TCID50 ml-1 TGEV (100µl/well). Plates were further incubated for 2hrs; virus supernatants were discarded and unbound virus was washed off with 1% PBS (2x) and replaced with DMEM (supplemented with 1% FCS and 1% penicillin/ streptomycin). Plates were incubated at 37°C for 48hrs and anti-viral effect of the probiotic was determined by doing MTT assay on the 96-well cell cultures. Briefly, 20µl of 5mg/ml MTT (Sigma Aldrich) was added to each well and plates were further incubated at 37°C in a humidified 5% CO_2 incubator for 4hrs. The medium was replaced with 100µl of dimethyl sulphoxide containing 0.6% acetic acid and 100g sodium dodecyl sulfat (SDS) (Sigma Aldrich). SDS solution and the cell lysates were then read in an ELISA plate reader (Tecan) at 590nm. All experiments were done in triplicates.

2.3.7.4. Virus titre (TCID50) determination

Washed monolayers of type II cells on 6-wells plates were incubated with 1 x 10^6 viable probiotic bacteria suspended in 1ml DMEM or 1ml probiotic culture supernatant diluted 1:1 in DMEM for 2hrs at 37°C in the atmosphere of 5% CO_2. After incubation, 50µg/ml of gentamicin in DMEM was added and plates were further incubated for 1hr. Monolayers were washed (2x) with DMEM (without supplements) and challenged with $10^{7.7}$ $TCID_{50}$ ml-1 TGEV (1ml/well). Monolayers were incubated for 2hrs, washed twice with PBS (1%) and about 3ml of DMEM supplemented with 1% FCS and 1% penicillin/ streptomycin and 20µg/ml of gentamicin was added to each well. Plates were incubated at 37°C for 48-72hrs and virus supernatant was collected by repeated freezing and thawing of the culture plates. The viral titre of triplicate experiments was expressed in $TCID_{50}$ values as described above.

2.3.7.5. Determination of cytokine modulation by the probiotic bacteria

2.3.7.5.1. Pre-treatment and harvest of cells after virus infection

Type II cell monolayers on 6-wells plates were incubated with 1 x 10^6 viable probiotic bacteria suspended in 1ml DMEM or 1ml of filter-sterilized probiotic culture supernatant diluted 1:1 in DMEM for 2hrs at 37°C in the atmosphere of 5% CO_2. For control plates only 1ml DMEM was added. After incubation 50µg/ml of gentamicin in DMEM was added and plates were further incubated for 1hr. Monolayers were washed twice with DMEM (without supplements) and challenged with $10^{7.7}$ $TCID_{50}$ ml-1 TGEV (1 ml/well). In the case of control plates only non-supplemented DMEM was added. Following incubation for 2hrs, monolayers were washed twice with PBS and ca. 3ml of DMEM supplemented with 1% FCS and 1% penicillin/ streptomycin and 20µg/ml of gentamicin was added to each well. Cells were collected by trypsinizing plates after 2hrs and 48hrs incubation at 37°C in 5% CO_2 atmosphere. Cells were washed twice with PBS (1%) and centrifuged at 300 x g for 10min.

2.3.7.5.2. Total RNA extraction and reverse transcription

Total RNA from the above cell pellets was isolated using Invisorb Spin Cell RNA Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. The amount and quality of RNA was determined using nanodrop system. As described above, the RNA samples were treated with DNase and reverse transcription was carried out with oligonucleotide dT and MMLV reverse transcriptase using the RevertAid™First Strand cDNA Synthesis kit (Fermentas, Germany) according to the manufacturer's protocol. The

reverse transcription was done at 42°C for 60min followed by 10min at 70°C for termination of the reaction. The obtained cDNAs (20µl) were stored at –20°C until use.

2.3.7.5.3. Real time PCR

In preliminary experiments, expression of the investigated factors (ß-actin, IL-6, IL-8 and IFN-γ) was examined by standard RT-PCR as described above. The sequences of the primers used and their optimal annealing temperatures are described on Table 4. The effect of the probiotic pre-treatment on the relative gene expression of the selected cytokines was studied by Real-time PCR using iCycler iQ5 real-time PCR detection system (Bio-Rad) and the SensiMix Plus SYBR kit (Quantace). Five hundred nano-gram cDNA was used as template for the real-time PCR containing 10µl of SYBR Green I Supermix (Bio-Rad) and 0.4µl (10pM/ml) of each primer (forward and reverse) in a final volume of 20µl. The quantitative real-time PCR mixtures contained 10µl of SYBR Green, 0.4µl of primer (10pM/ml), in a final volume of 20µl.

The following real-time PCR protocol was applied: initial denaturing step at 94°C for 3min, three-step amplification (50x), including denaturation at 94°C for 20sec, annealing 53-55°C for 20sec and extension at 72°C for 5min. An end-point single fluorescence was measured after each extension step. The melting curves for PCR products were analysed from 70 to 95°C to determine the specificity of all amplifications. The obtained melting points of the amplified products served as conformation for specific amplification. As negative controls, reactions containing no template (H_2O) or no cDNA were included to verify that obtained PCR products were not derived from contaminations or genomic DNA.

Calculations were performed using the Bio-Rad iQ5 software by normalizing the relative amounts of each gene to the internal β-actin control in the respective sample. The difference in the expression of genes between TGEV infected samples with and without probiotic pre-treatment was statistically compared using SPSS (version 14). *In vivo*, Kruskal-Wallis test was used to compare the differences attributed to the probiotic supplement and Mann-Whitney U test was performed to compare the differences at a specific time after *Salmonella* infection. *In vitro*, analysis of variance was used to compare differences between the various groups of experiments. In all cases, differences with $P < 0.05$ were considered significant.