3.0. RESULTS

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

3.1.1. Percentage of CD8 positive lymphocytes in the probiotic and control groups

The percentages of CD8+ lymphocytes in the IEL isolated from the jejunum of piglets of the probiotic and control groups 24h, 71h and 28d after *Salmonella* infection were analysed using flow cytometry. Piglets of the probiotic group had significantly lower percentage of CD8+ cells in the IEL 24h after *Salmonella* infection. The differences in the percentages CD8+ cells 71h and 28d after infection were not statistically significant ($P > 0.05$) (Fig.12). The percentages of CD4+ cells in the spleen (Fig.13A) and proximal PP (Fig. 13B) of both groups showed no significant variation between the probiotic and control groups.

Percentages of CD8+ lymphocytes in the probiotic and control groups

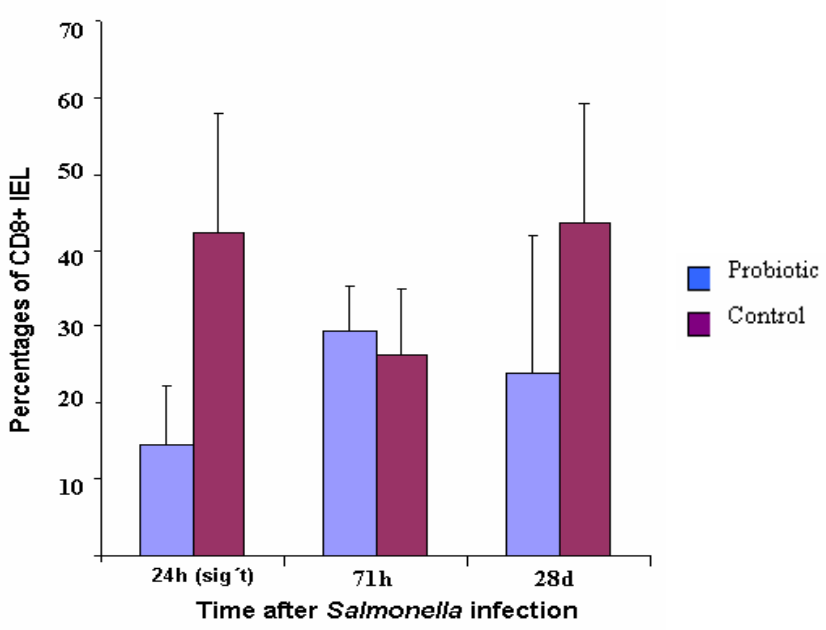


Fig. 12. *The percentage of CD8+ lymphocytes in the IEL of the probiotic and the control groups was measured by FACS. Piglets of the probiotic group had a significantly lower ($P < 0.05$) percentage of CD8+ lymphocytes in the IEL 24h after Salmonella infection. The differences in the percentages of CD8+ lymphocytes at the other time points after infection were not significant ($P > 0.05$). The error bars represent the standard deviations of the measurements.*

3.1.2. Percentage of CD4 positive lymphocytes in the probiotic and control groups

The percentages of CD4+ lymphocytes in the discrete PP of piglets of the probiotic and control groups were analysed 24h and 28d after *Salmonella* infection using FACS. The variations in the percentages of CD4+ lymphocytes in the discrete PP both at 24h and 28d after infection were not significant. However, at 28d post infection, there was a tendency towards higher percentages of the lymphocytes in the probiotic group (Fig. 13). In spleen lymphocytes, the percentage of CD4+ cells 3h, 71h and 28d after *Salmonella* infection showed tendencies towards higher percentages in the piglets of the probiotic group than the control. However, none of these significances were statistically significant.

Percentages of CD4+ lymphocytes in the probiotic and control groups

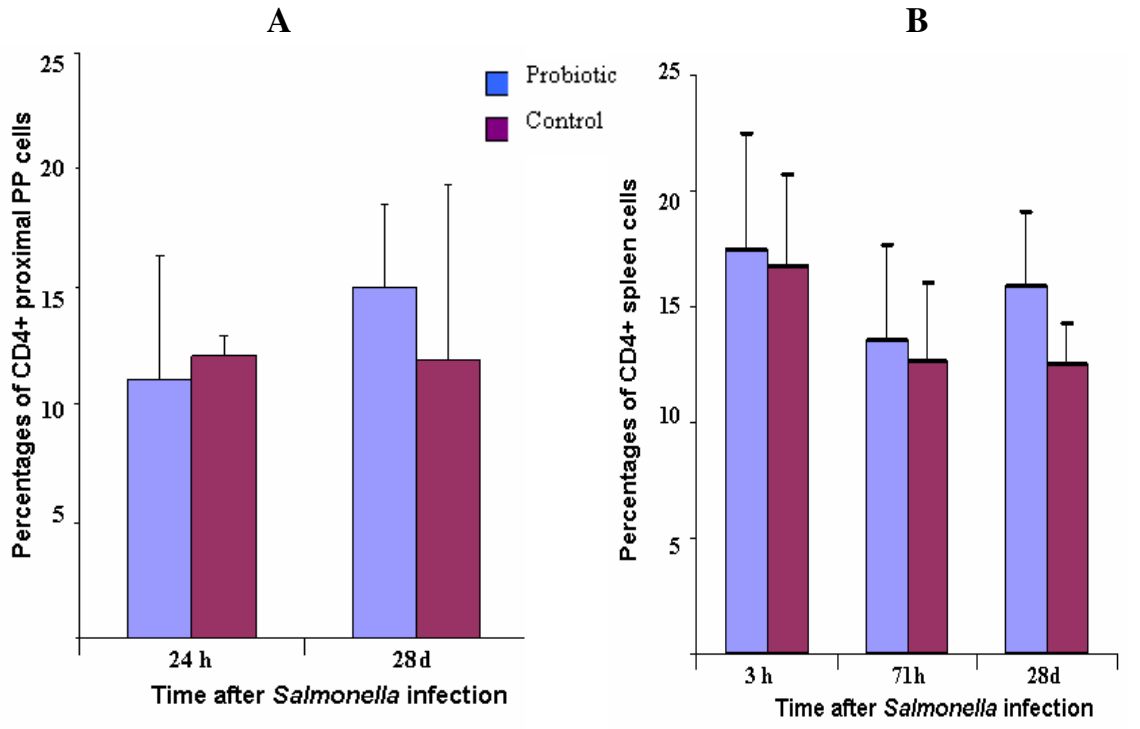


Fig. 13. *The percentage of CD4+ lymphocytes in the discrete PP (A) and spleen (B) of the probiotic and the control groups was measured by FACS. There was no significant ($P > 0.05$) difference in the percentage of CD4+ lymphocytes in both organs of piglets of the probiotic and control groups at each time point after Salmonella infection. However, there was a tendency towards a higher percentage of CD4+ lymphocytes in the probiotic group, especially at 28d after Salmonella infection. The error bars represent the standard deviations of the measurements.*

3.1.2. Isolation of CD4+ and CD8+ lymphocytes by MACS

The isolation of CD4+ cells from the discrete PP and spleen of piglets of the probiotic and control groups using the MACS method was successful. Before isolation of the specific cells from the total lymphocytes, only 10-20% of the cells isolated from the spleen (Fig. 14) and the discrete PP (not shown) were CD4+. In the IEL samples, 30 to 50% of the total lymphocytes were CD8+ (Fig. 15A). After isolation of the specific lymphocytes using MACS, 85 to 96% of the cells isolated from the spleen (Fig. 14B) and discrete PP (not shown) were CD4+ lymphocytes. Similarly, 86 to 97% of the total lymphocytes isolated from the IEL were CD8+ (Fig. 15B).

Percentage of CD4+ cells before and after MACS

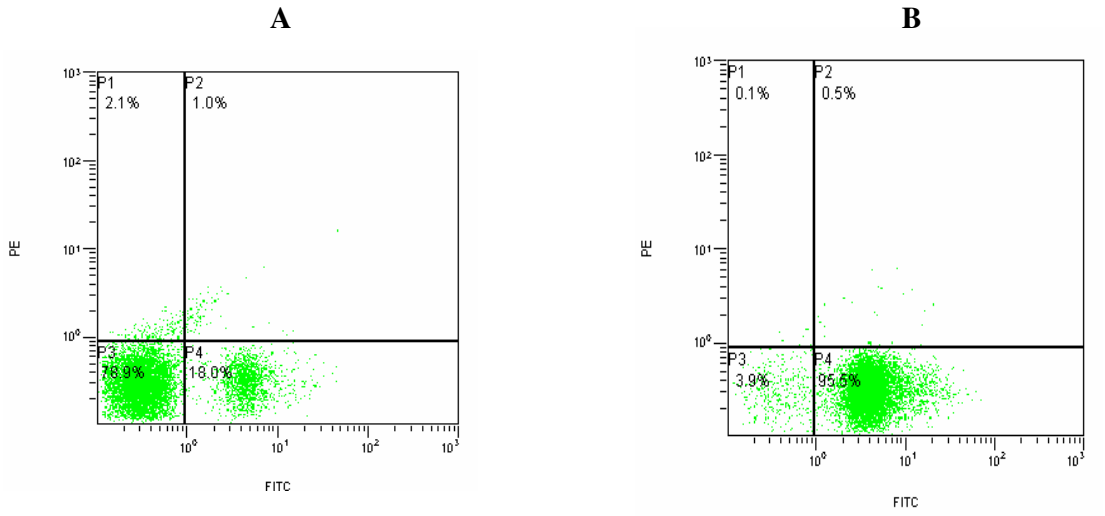


Fig. 14. *The percentage of FITC-conjugated CD4+ lymphocytes from the total lymphocytes isolated from a spleen of a piglet before (A) and after (B) isolating CD4+ cells using MACS. Only 18% of the total lymphocytes were CD4+ before MACS purification. After isolation was done by MACS method, 95.5% of the total lymphocyte population in the spleen were CD4+.*

Percentage of CD8+ cells before and after MACS

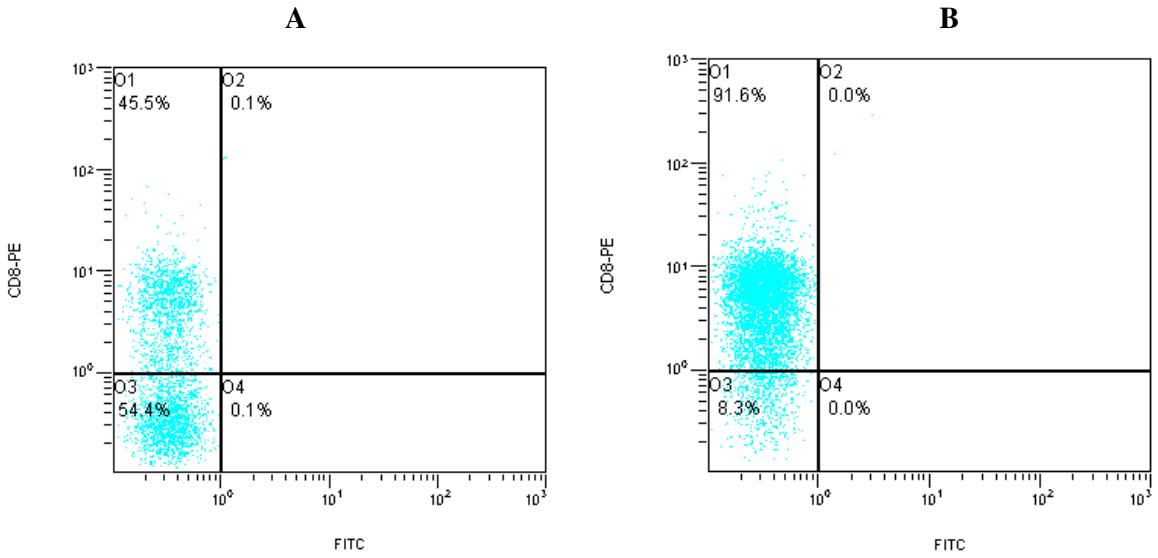


Fig. 15. *The percentage of PE-conjugated CD8+ lymphocytes from the total lymphocytes isolated from the IEL of a piglet before (A) and after (B) isolating CD8+ cells using MACS. Only 45.5% of the total lymphocyte population were CD8+ before MACS was performed. After MACS purification was performed, 91.6% of the total lymphocyte population were CD8+.*

The relative expression of genes in the spleen, discrete proximal PP and IEL samples could not be studied using real-time PCR due to the small number of cells (ca. 1x 10^5 cells/sample) obtained after isolation using MACS. Below are shown the results of the gene expression analysis in the PBMC and distal PP of piglets of the probiotic and control groups. All the data are represented using dot plots, where the lines in the boxes represent the 25^{th} percentile (first quartile), the 50^{th} percentile (the median) and the 75^{th} percentile (the third quartile); such as the percentiles represent the number below which 25%, 50% or 75% of the data points, respectively, fall. The graphs also show the sample identity numbers that are outliers; which means that the samples have values that are either smaller than the lower quartile minus 1.5 times the inter-quartile range (inner box) or larger than the upper quartile plus 1.5 times the inter-quartile range.

3.1.3. IL-1 gene expression in PBMC

The mRNA expression of the genes for IL-1ß and IL-1α in PBMC of the piglets was significantly affected by the probiotic treatment. Irrespective of the time after infection, the relative gene expressions of IL-1ß and IL-1α were significantly higher ($P < 0.05$) in the PBMC of the probiotic group than the control group. With respect to the time after *Salmonella* infection, the differences in the expressions of the above genes at 71h after *Salmonella* infection were significantly higher in the probiotic group than the control group ($P < 0.05$) (Fig. 16). No significant differences ($P > 0.05$) in the levels of expressions of the above genes were observed at 3h and 28d after *Salmonella* infection ($P > 0.05$) (Fig. 16).

Effect of *Enterococcus faecium* SF68 on IL-1ß and IL-1α at the gene level

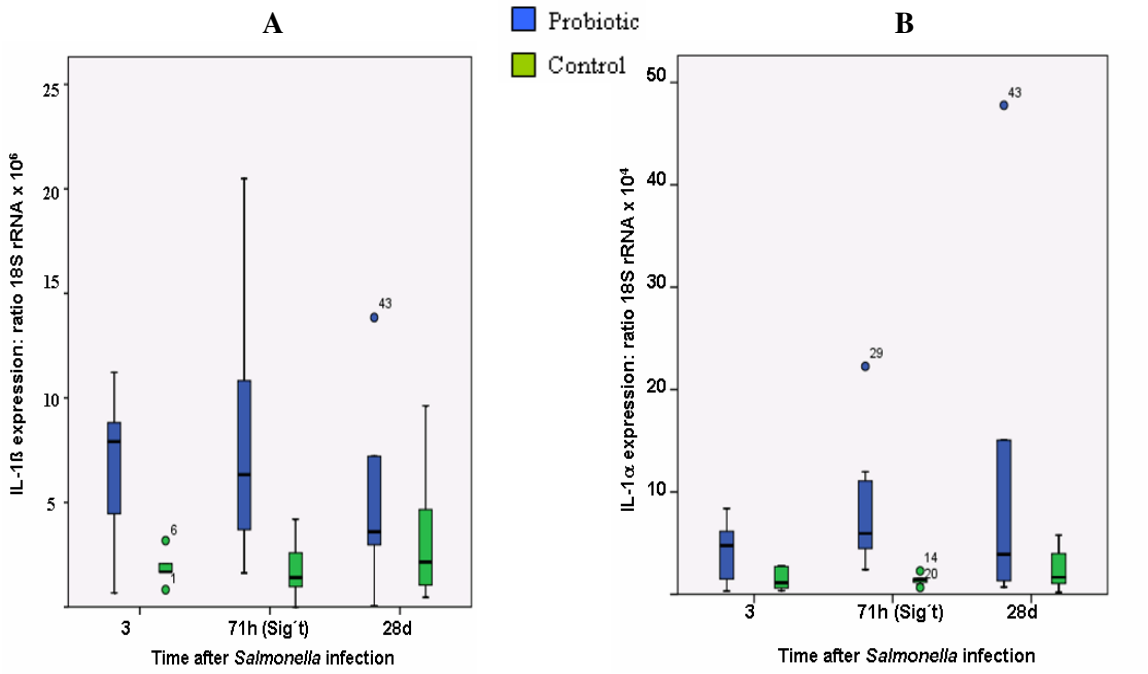


Fig. 16. *The mRNA expression levels of IL-1ß (A) and IL-1α (B), as measured by real-time PCR, were significantly affected by the probiotic treatment. Piglets of the probiotic group had a significantly higher expression of both cytokines in the PBMC 71h after Salmonella infection. The difference in the expression of the genes was not significant 3h and 28d after infection. Irrespective of the time after Salmonella infection, the genes for both IL-1ß and IL-1α were significantly up-regulated in the PBMC of piglets of the probiotic group as compared with those in the control group.*

3.1.4. IL-8 gene expression in PBMC

The mRNA expression of IL-8, which is also associated with inflammatory reactions, was also affected by the probiotic supplement. Irrespective of the time after infection, the relative gene expression of IL-8 was significantly higher ($P < 0.05$) in the PBMC of the probiotic group than the control group (data not shown). The expression of the above gene at 71h after *Salmonella* infection was significantly higher ($P < 0.05$) in the probiotic group than the control group (Fig. 17). The differences in the level of expression of this gene between the two groups 3h and 28d after *Salmonella* infection was not significant ($P > 0.05$) (Fig. 17).

Effect of E. faecium SF68 supplement on the expression of IL-8

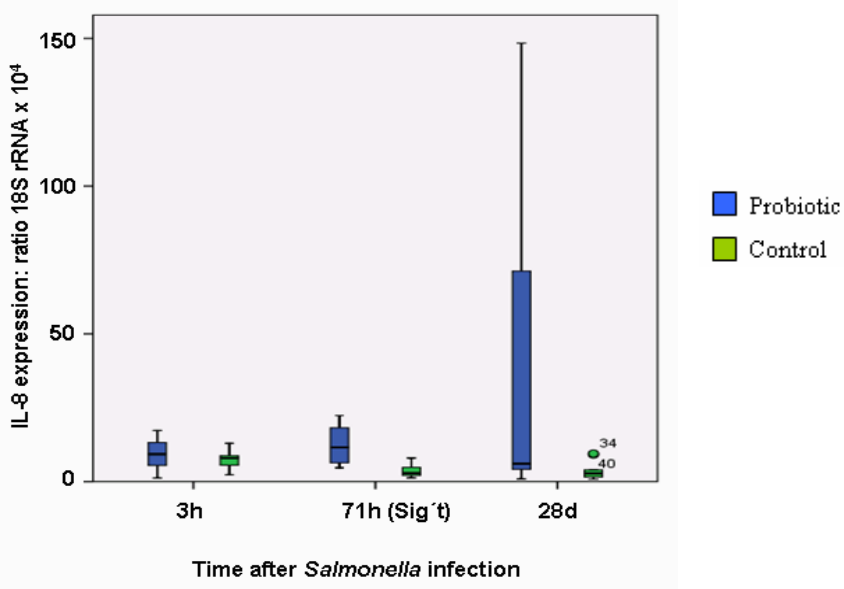


Fig. 17. *In the PBMC, piglets of the probiotic group had a significantly higher expression of the mRNA for IL-8 71h after Salmonella infection ($P < 0.05$). At 3h and 28d after infection, the difference in the expression of the gene was not significant ($P > 0.05$).*

3.1.5. TLRs gene expression in PBMC

The effect of the probiotic supplement on the mRNA expressions of the toll-like receptors (TLRs), TLR2 and TLR9, was also examined using real-time PCR. Similarly, our results showed that the piglets of the probiotic group had significantly higher levels of expressions of the mRNA for TLR2 and TLR9 in the PBMC 71h after *Salmonella* infection (Fig. 18). The general expressions of both genes throughout the infection period were also significantly higher in the probiotic than the control group ($P < 0.05$). The mRNA expressions of both genes did not significantly vary at 3h and 28d after *Salmonella* infection ($P > 0.05$) (Fig. 18).

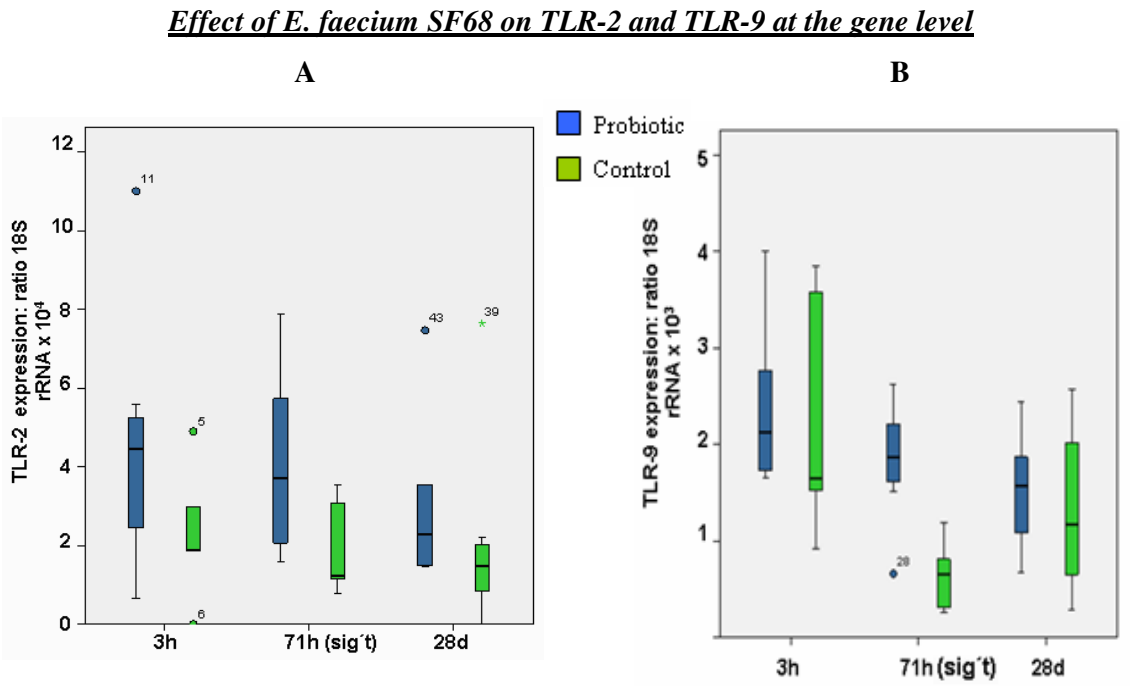


Fig. 18. *The expressions of mRNA to TLR-2 (A) and TLR-9 (B) were significantly affected by the probiotic treatment. Piglets of the probiotic group had a significantly higher expression of both receptors in the PBMC 71h after Salmonella infection. The difference was not significant 3h and 28d after infection. Both genes were significantly higher expressed in the probiotic group than the control group through out the infection period.*

3.1.6. IL-1ra gene expression in PBMC

The expression of IL-1ra in the PBMC of the probiotic group 71h after infection was much higher (P = 0.093) than that in the control group. The general mRNA expression of the gene, irrespective of time after *Salmonella* infection, was also higher in the probiotic group than the control group (p=0.092). However, none of these differences was statistically significant.

Effect of Enterococcus faecium SF68 on the expression of IL-1ra

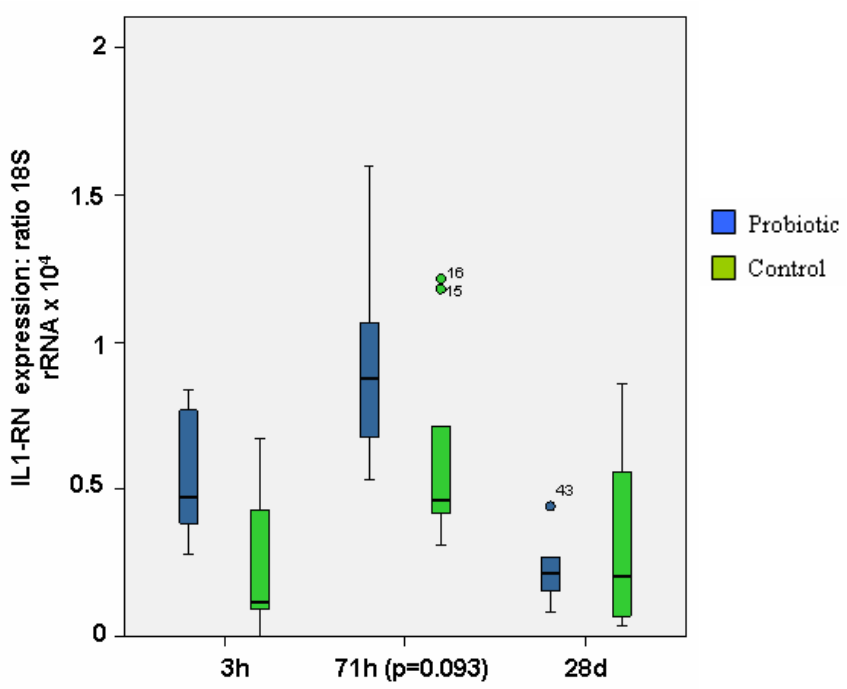


Fig. 19. *Real-time PCR analysis for the mRNA expression of IL-1ra (IL-1RN) in PBMC. The probiotic treatment up-regulated the expression of IL1-ra in piglets of the probiotic group irrespective of the duration after Salmonella infection (p=0.092). The up-regulation was more pronounced 71h after infection (p=0.093). However, none of these up-regulations were found to be statistically significant.*

3.1.7. TGF-ß, CD-9 & other gene expressions in PBMC

Our real-time PCR analysis also showed that the general mRNA expression of the genes for TGF-ß and CD-9 were significantly higher in the PBMC of piglets of the probiotic group than those of the control group ($P < 0.05$) (Fig. 20). The differences in the expression levels of both genes at each time point after *Salmonella* infection appeared to be statistically insignificant.

Our expression analysis for the mRNA various genes also showed that the probiotic supplement did not have a significant effect on the mRNA expressions of IL-2, IL-6, IL-10, TNF-α and IFN-γ in the PBMC (data not shown).

Effect of Enterococcus faecium SF68 on the level of expressions of TGF-ß and CD-9

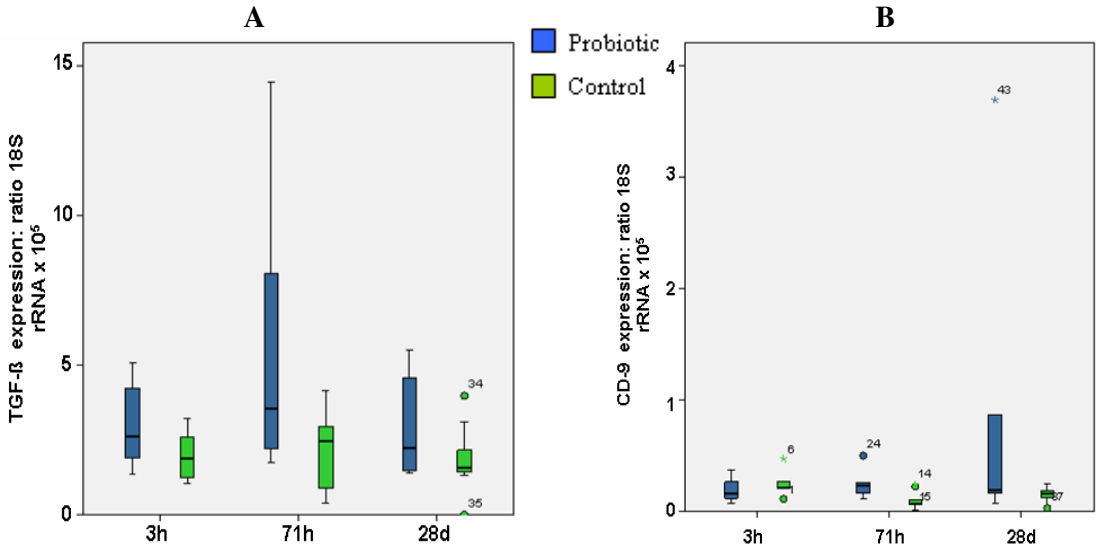


Fig. 20. *The general expressions of the mRNA of TGF-ß (A) and CD-9 (B) in PBMC of the piglets was significantly higher in piglets of the probiotic group ($P < 0.05$); however, the effect at each time after Salmonella infection was not varying significantly ($P > 0.05$).*

3.1.8. Gene expressions in distal continuous PP

In the distal PP, none of the above genes, except IL-1α, were significantly affected by the probiotic supplement (data not shown). The mRNA expression of IL-1α was significantly ($P < 0.05$) up-regulated in the distal continuous PP of piglets of the probiotic group at 24hrs after *Salmonella* infection (Fig. 21).

Effect of E. faecium SF68 on the level of expression of IL-1α in distal PP

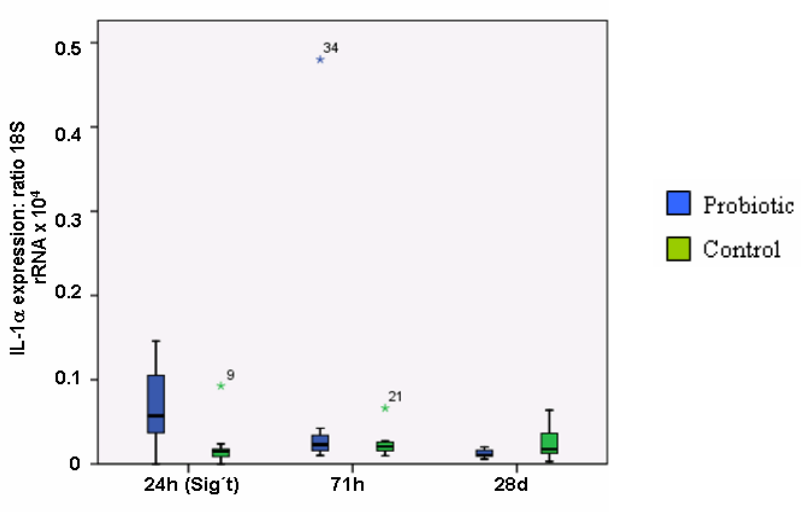


Fig. 21. *In piglets of the probiotic group, the expression of IL-1α in the distal PP was significantly ($P < 0.05$) up-regulated 24h after Salmonella infection. The difference in the gene expression of the cytokine was not significant at 71h and 28d after Salmonella infection.*

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

3.2.1. Difference of type I and type II cells in cell-size and growth rate

The characterization of previously isolated pig intestinal cells, which we referred as type I and type II cells, started with microscopy. The phase-contrast microscopy of the cells at their 50th passage is shown on Figure 22. Both cells grow well in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin and did not change their primary morphologies through the number of passages. Both cells survived for more than 14 days in a 3D culture but they did not increase in number. Type I and type II cells have distinct morphology in that type I cells are smaller and have uniform columnar shape. Type II cells have irregular shape and are bigger than type I cells. The distribution of both cells on a dot-plot of FSC versus SSC after FACS measurement showed that type II cells are more heterogeneous in shape and size than type I cells (Fig. 23). Most type II cells resulted in greater SSC than type I cells, indicating that type II cells are larger than type I cells. There is no much difference in the amount of FSC light scattered by both cells. This implies that the cells do not differ much in cell granularity.

Phase-contrast microscopy of type I and type II cells

A **B**

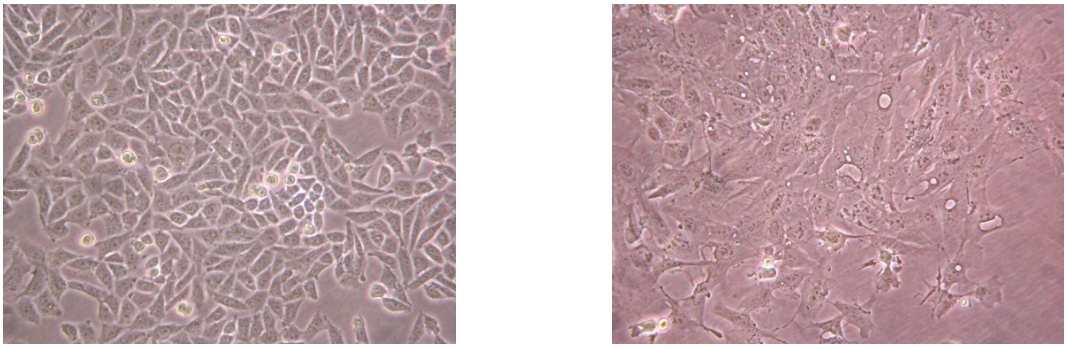


Fig. 22. *Phase-contrast microscopy (400x) of type I (A) and type II (B) cells at their 50th passage in DMEM medium supplemented with 10% FCS and 1% penicillin-streptomycin. Type I cells form single layer of columnar cells while type II cells have single layer of irregular shaped cells, which are relatively larger in size.*

FACS analysis of type I and type II cells for differences in size and homogeneity

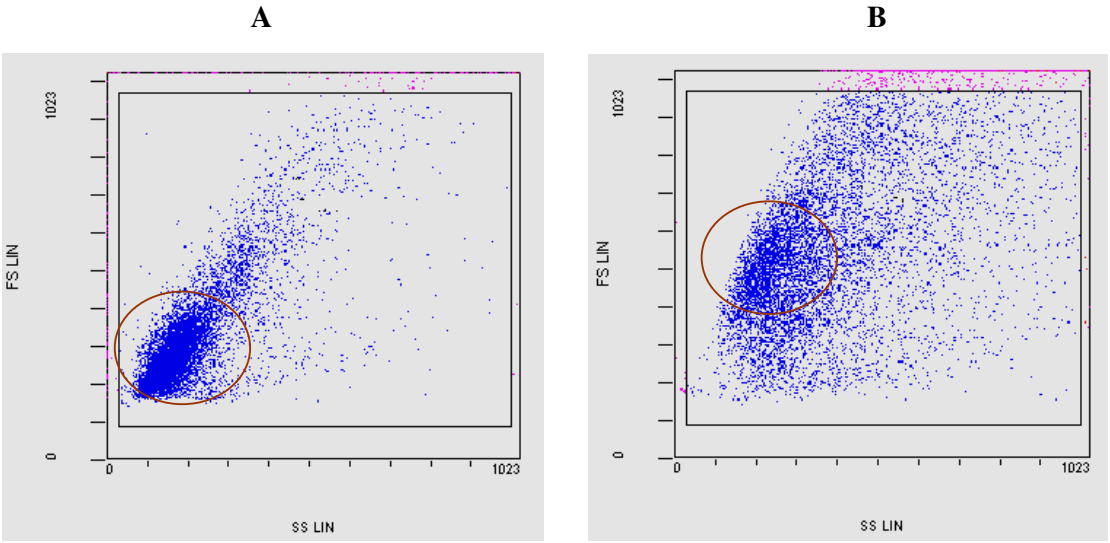


Fig. 23. *Type II cells (B) were more scattered than type I cells (A) on a dot-plot made from SSC verses FSC light after FACS analysis, showing that type II cells have a more heterogeneous morphology. Most type II cells (in the circled region) are larger than type I cells, which is proportional to the FSC light. There is no much difference in the granularity of the cells, which is proportional to the SSC light.*

Light microscopy of semi-thin sections made from monolayers of type I and type II cells grown on a collagen-coated culture plate before electron microscopy also confirmed that type I cells are smaller than type II cells and that both cells form a monolayer.

Light microscopy of semi-thin-sections of type I and type II cells

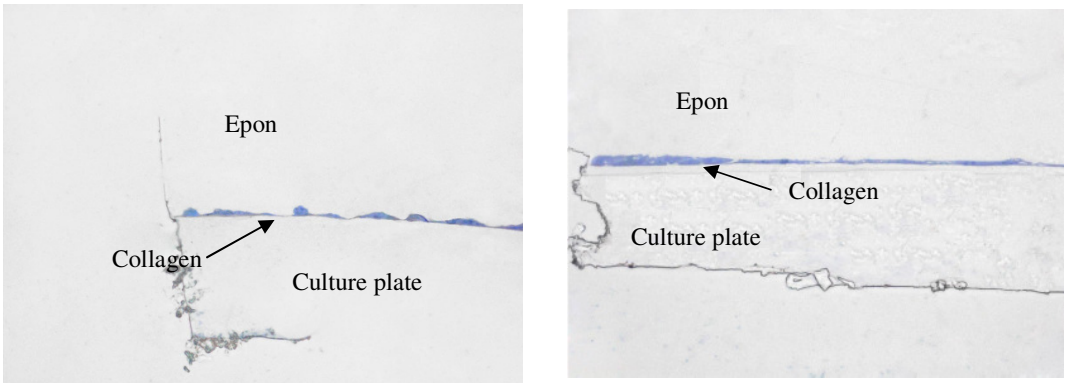


Fig. 24. *Light microscopy of semi-thin sections of monolayers of type I (A) and type II (B) cells grown on collagen-sheathed culture plates. Both cells form monolayers and type I cells are smaller in size than type II cells.*

Although DMEM supplemented with 10% FCS and 1% penicillin/streptomycin supports the growth of both type I and type II cells, type I cells have a faster rate of growth than type II cells that we needed to trypsinized a culture flask ($25mm^3$) twice a week. In contrast, type II cells needed ca. 7 days to reach confluency. The generation (doubling) time of type II was 48h while that of type I cells was 24hrs, twice faster (Fig. 24).

Growth curves of type I and type II cells

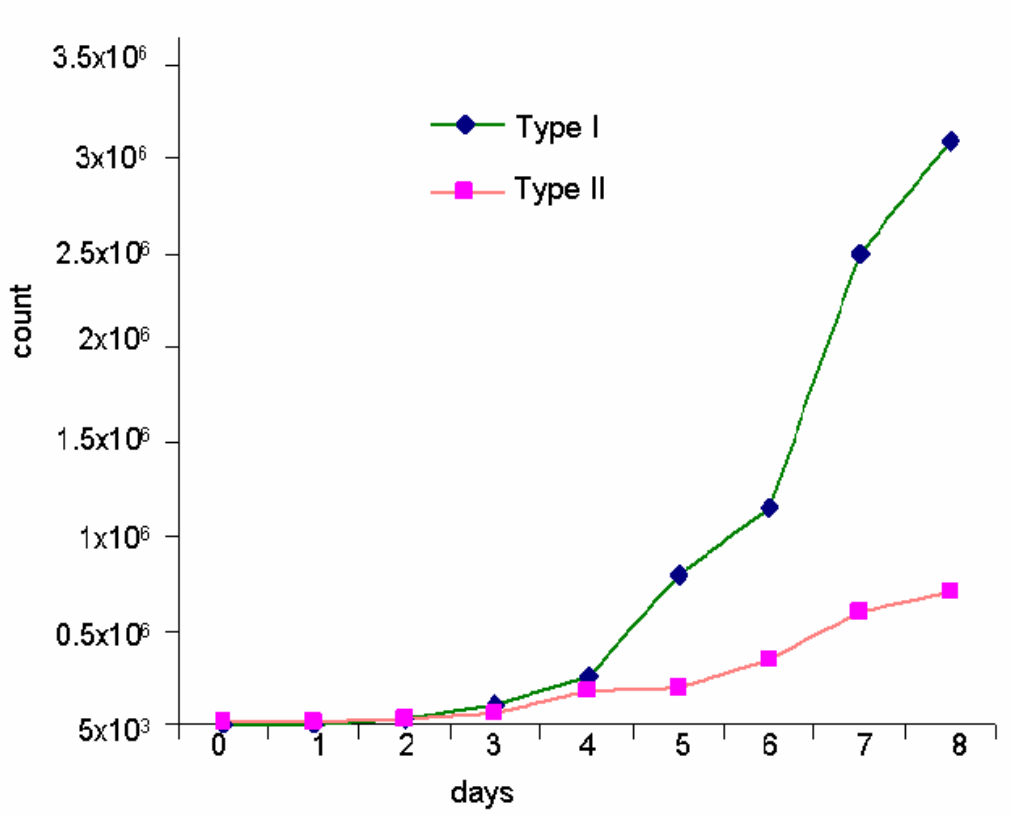


Fig. 24. *Type I and type II cells were grown in DMEM supplemented with 10% FCS and 1% Penicillin/streptomycin in 24-wells plates and three wells were trypsinized and counted every day, from which the average counts are indicated. The growth curves show that type I cells grow twice faster than type I cells. The generation time of type I and type II cells was 24d and 48d, respectively.*

3.2.2. Overviews of type I and type II cells under transmission electron microscope (TEM)

Electron microscopic observations of type I and type II cell cultures showed that the cells have distinct morphological features. Both cells have large nuclei that are irregular in shape but the nuclei of type II cells appeared to be more folded. Both cells exhibited microvilli on their surfaces and between cells stretching parallel to each other (Fig. 25 & 26).

Overviews of type I and Type II cells under TEM

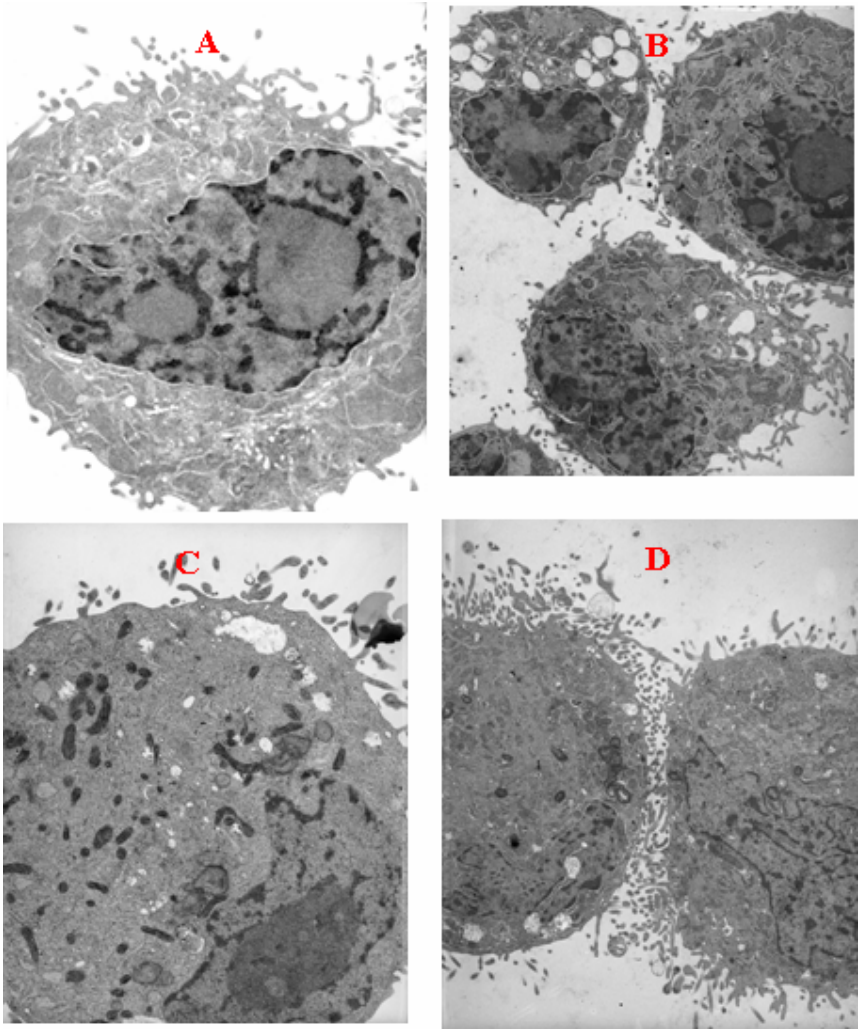


Fig. 25. ***TEM of single cells of trypsinized type I (A & B) and type II (C & D) cells.*** *Both cells have large nuclei but the nuclei of type II cells are more folded. The surfaces of both type I and type II cells are covered with microvilli.*

3.2.3. The ultra-structures of type I and type II cells

3.2.3. The microvilli

Microvilli strongly increase the cell surface area and thus raise resorption in intestinal epithelial cells. The presence of microvilli both in type I and type II cells indicated their epithelial nature. The microvilli of type I cells had microfilaments, which were not visible in type II cells (Fig 26 & 27). The microvilli both type I and type II cells do not have a developed terminal web like those in the absorptive epithelial cells (Fig 26).

Microvilli of type I and type II cells

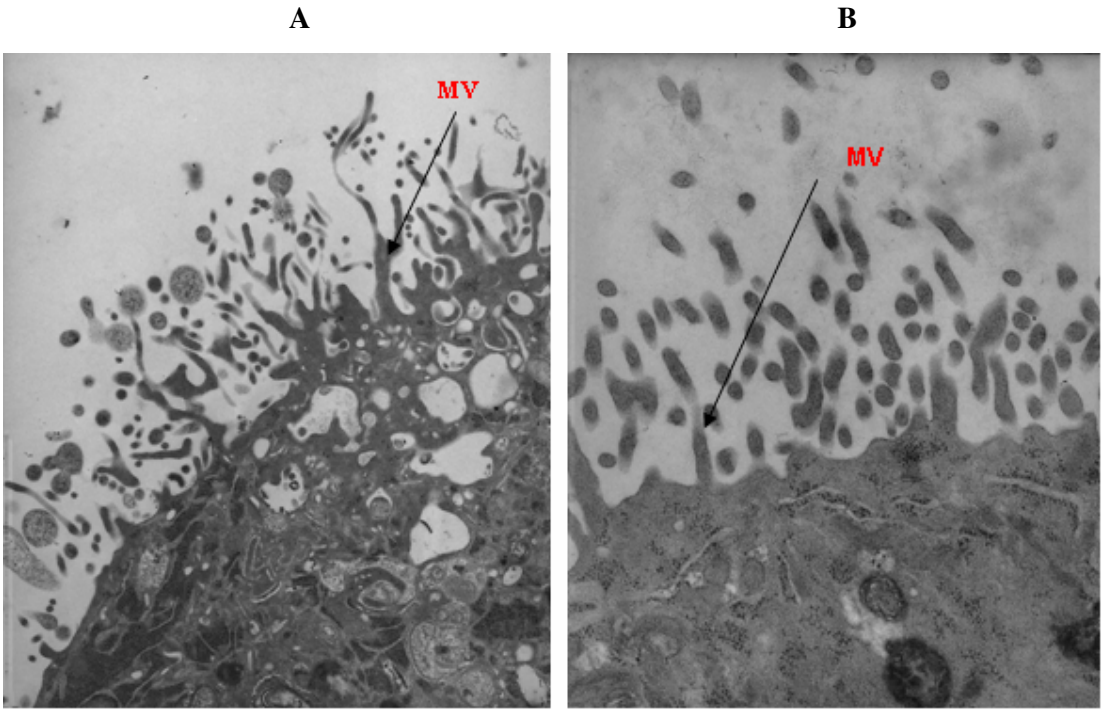


Fig. 26. ***TEM of the microvilli (MV) of type I (A) and type II (B) cells.*** *Both cell types are characterized by a number of microvilli covering their surfaces. The microvilli of type II cells do not contain microfilaments (MF), which were seen in type I cells (see also Fig 27). The microvilli of both type I and type II cells do not have a developed terminal web.*

3.2.4. Cell-to-cell contact

We could observe cell-to-cell contacts in both type I and type II cells both from trypsinized and non-trypsinized cell preparations (Fig. 27). Type I cells were interconnected with one another but desmosomes were not detected (Fig 27). Cell-to-cell contact was also observed in trypsinized type II cells; however, in non-trypsinized monolayers, no cell-to-cell contact was

seen between type II cells, except that the cells were close to one another. Tight junctions (Tj), which are predominantly found in absorptive epithelial cells, were not seen in both type I and type II cells.

TEM micrographs of cell to cell contact in type I and type II cells

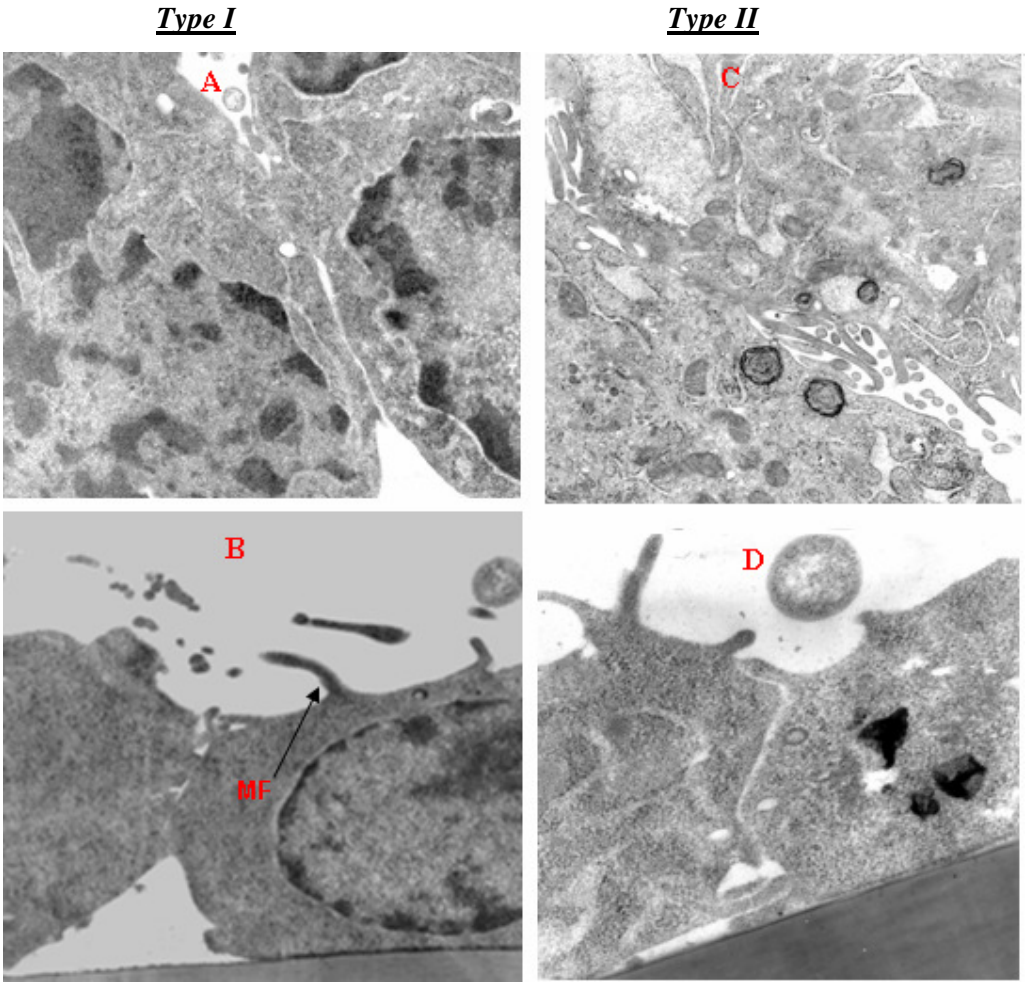


Fig. 27. *Cell-to-cell contact in type I (A & B) and type II cells (C & D). A & C are TEM pictures of type I and type II cells, respectively, after the cells were trypsinized and B & D are TEM pictures from non- trypsinized monolayers of type I and type II cells, respectively. Although cell-to-cell contact was seen tight junctions (Tj) or desmosomes are not detected in both cell types.*

3.2.5. Organelles

Type I and type II cells have common ultra-structural features such as the endoplasmic reticulum (ER), Golgi complexes and mitochondria, with dense matrixes. The inner and the outer membranes of the mitochondria and the cristae (cr) of the mitochondria are visible in both types of cells. However, type II cells have characteristic extremely elongated mitochondria and a number of well-developed multilaminar bodies (MB) (Fig. 29). The apical cytoplasm of type II cells is relatively more densely packed with organelles and their nuclei appear to be more folded than those of type I cells (Fig. 25 & Fig. 29).

TEM micrographs of the different organelles in type I cells

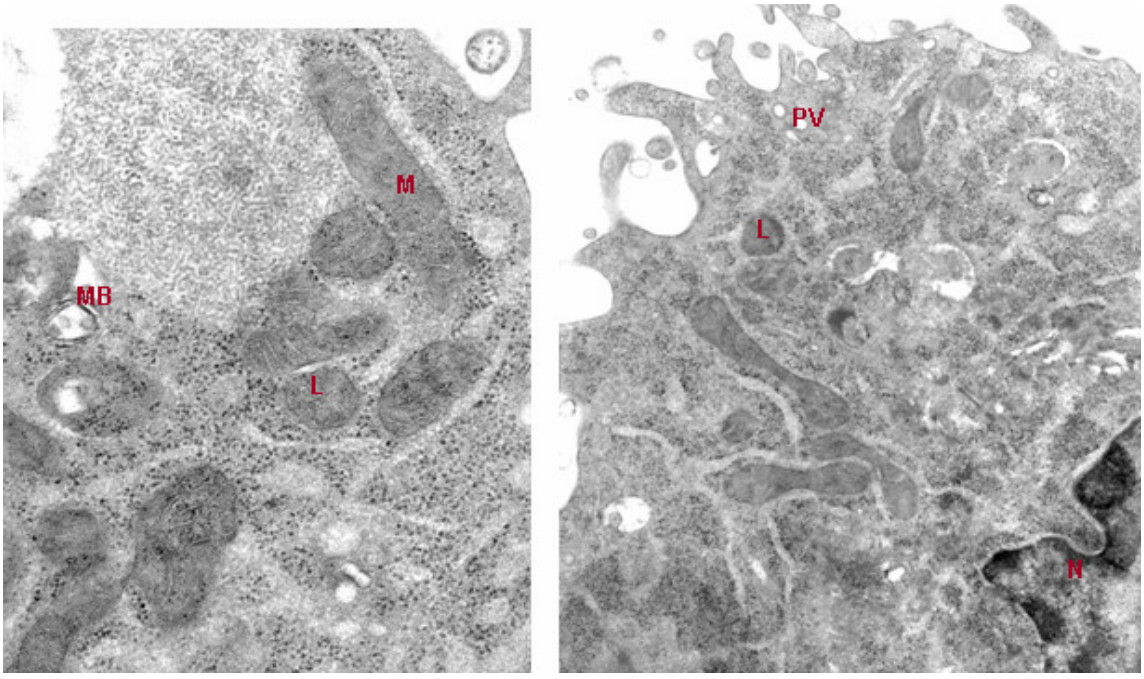


Fig. 28. ***Organelles in type I cells.*** *In addition to the nucleus, a few organelles, such as electro-dense mitochondria (M), multilaminar bodies (MB) and lysosomes were observed in Type I cells. The MB in type II cells were not well-developed as in type II cells (Fig. 29). The mitochondria of type II cells were also not as big as those in type II cells. In addition, the cytoplasm of type I cells was less populated with pinocytic vesicles (PV), Golgi apparatus (G), smooth and rough endoplasmic reticulum (SER & RER) as compared to that of the type II cells (Fig. 29). However, type I cells have relatively more abundant lysosomes (L) as compared to type II cells.*

TEM micrographs of the different organelles in type II cells

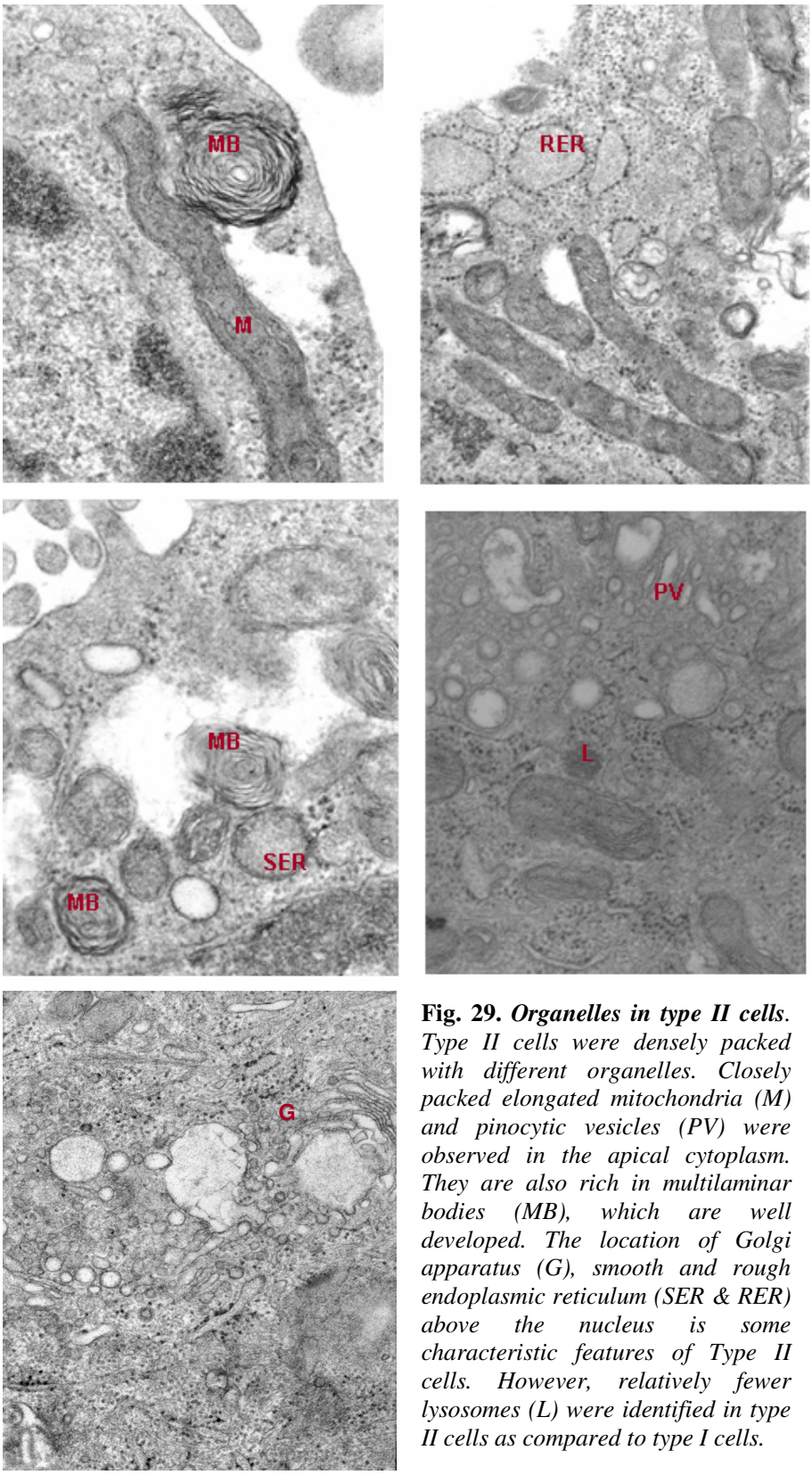


Fig. 29. ***Organelles in type II cells***. *Type II cells were densely packed with different organelles. Closely packed elongated mitochondria (M) and pinocytic vesicles (PV) were observed in the apical cytoplasm. They are also rich in multilaminar bodies (MB), which are well developed. The location of Golgi apparatus (G), smooth and rough endoplasmic reticulum (SER & RER) above the nucleus is some characteristic features of Type II cells. However, relatively fewer lysosomes (L) were identified in type II cells as compared to type I cells.*

3.2.4. Studies on the expression of selected genes in type I and type II cells

The expression of various genes in type I and type II cells as studied by using RT-PCR is summarized in Table 5. Both cells express the mRNA for IL-1β, IL-6, IL-8, IL-10 and IFN-γ consistently. Both cells do not express Mucin 2, excluding their possibility to be goblet cells. Type I cells expressed of the mRNA for TNF-α, which was not the case in type II cells. On the other hand, type I cells expressed the mRNA for IL-2 and IL-4, while type II cells did not.

Table 5. Gene expression analysis of type I and type II cells

Gene expression		
Gene	**Type I cells**	**Type II cells**
IL-1β	+	+
IL-2	+	-
IL-4	+	-
IL-6	+	+
IL-8	+	+
IL-10	+	+
IFN-γ	+	+
TNF-α	-	+
Mucin 2	-	-

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

3.2.5.1. FACS analysis for CD14 and CD45 markers in type I and type II cells

Further characterizing type I cells and type II cells using immunohistochemical approach, we analyzed the expression of the macrophage marker, CD45 in both cells using FACS. Both type I and type II cells appeared to be negative for the markers CD14 and CD45 as shown on the histograms made after FACS analysis (Fig. 30). These results made the cells unlikely to be neither macrophages nor leukocytes.

FACS analysis for CD14 and CD45 markers in type I and type II cells

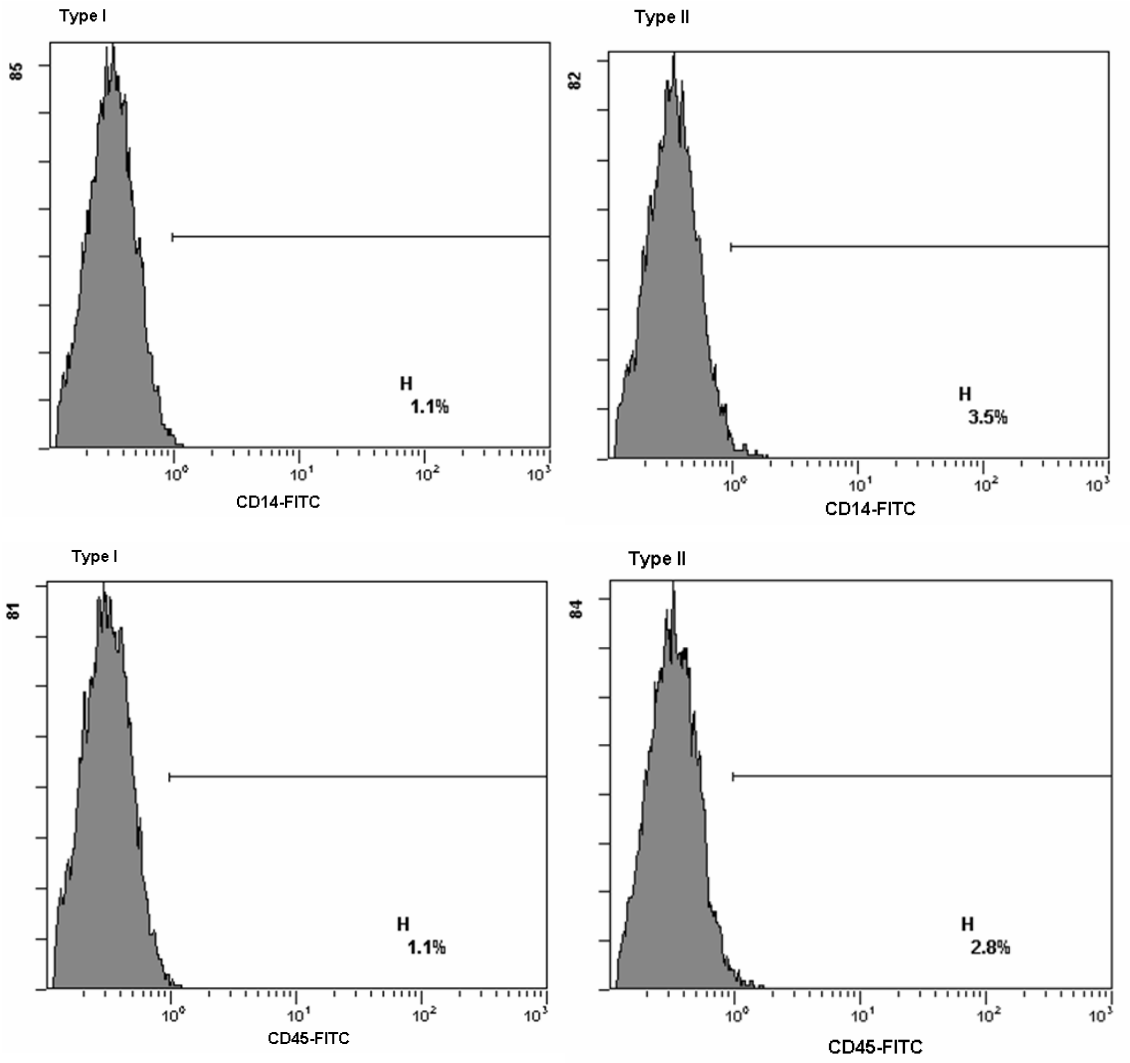


Fig. 30. *Histogram plots of type I and type II cells after incubating the cells with antibodies for CD14 and CD45 showed that both cells express neither of these markers.*

3.2.5.2. FACS analysis for endothelial markers

Furthermore, we used two different endothelial cell markers, von Willebrand factor (VWF) glycoprotein and CD31 (PECAM-1) for further FACS analysis. Our results showed that both cells did not express these markers; thus excluding the possibility of both cell types to be endothelial cells (Fig. 31).

FACS analysis for the Expression of von Willebrand factor (VWF) and CD31 (PECAM-1) in type I and type II cells

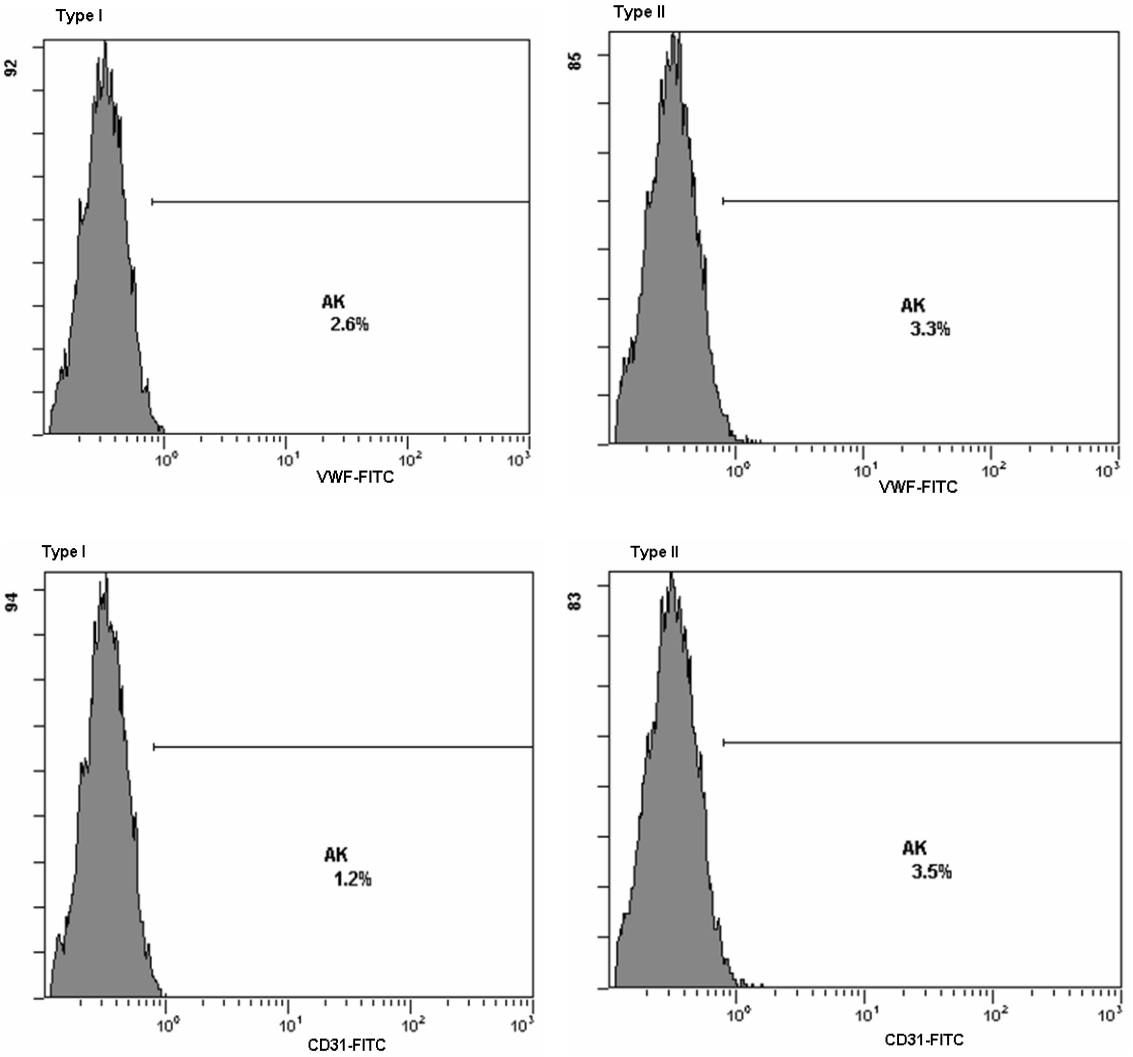


Fig. 31. *Histogram plots of type I and type II cells after incubating the cells with antibodies for von Willebrand factor (VWF) glycoprotein and CD31 (PECAM-1) show that both cells are negative for the markers, and thus not endothelial cells.*

3.2.5.3. Detection of an epithelial cell marker using Fluorescence microscopy

Finally, our histochemical characterization using the pan-cytokeratin marker gave a positive fluorescence both in type I and type II cells under a fluorescent microscope (Fig. 32). However, both cell types did not have a positive fluorescence for vimentin (data not shown). This, along with the ultra-structural morphology of both cells, particularly, the microvilli, confirmed the epithelial nature of the cells.

Expression of cytokeratin in type I and type II cells

Type I **Type II**

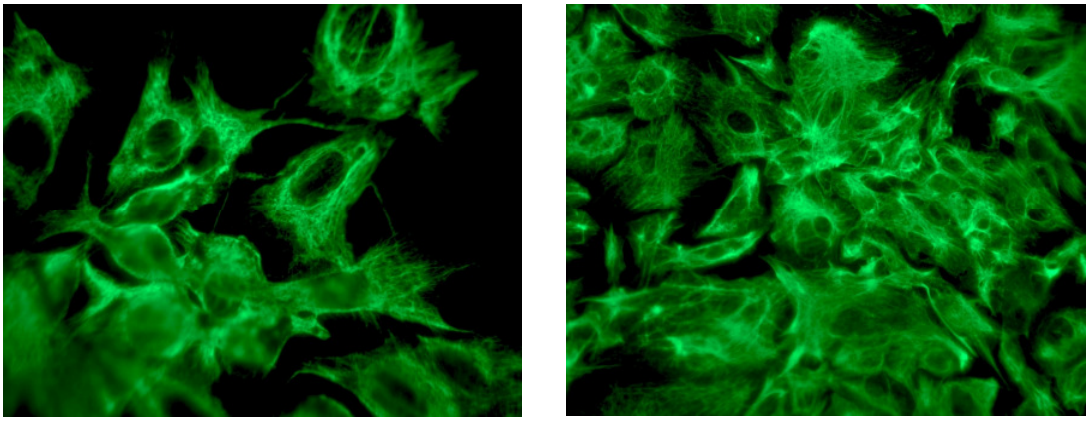


Fig. 32. *Detection of cytokeratin in type I and type II cells using a confocal laser-scanning microscope (Leica TCS SP2) after immunohistochemical staining. The green signal derived from the FITC-labeled pan-cytokeratin indicates that both cells are cytokeratin-positive cells, implying the epithelial nature of type I and type II cells.*

3.2.6. Sensitivity of type I and type II cells to TGEV infection

In addition to the differences in morphology and growth characteristics that we observed between type I and type II cells, it was interesting to notice that only type II cells were sensitive to the swine gastroenteritis virus, TGEV, which infects intestinal epithelial cells of the villous that have the receptor, amino-peptidase N (APN), for the viral antigenicity. Type I cells did not show any cytopathic effect (CPE) due to viral infection and maintained their morphology through out the infection period (3 days) (not shown). TGEV results in a progression of CPE in type II cells, whereby 48h after infection the cells attain a rounded morphology and the majority of the cells are detached from the culture plate (Fig. 33A) as the virus reaches its maximum titre (Fig. 33B).

Type II cells are sensitive to TGEV infection

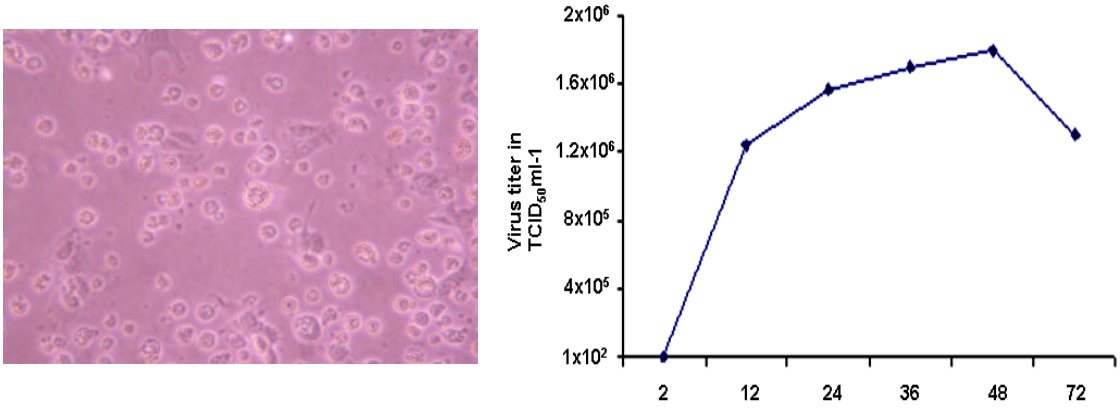


Fig. 33. *A phase-contrast microscopic image (A) showing cytopathic effect (CPE) of TGEV on type II cells 48h after infection and the growth curve of the virus in type II cells (B) showing that the virus reaches its maximum titre 48h after infection.*

More interestingly, the virus titre of TGEV obtained after continually passing the virus in type II cells (4 times) is approximately 2 log higher ($10^{7.7}$per ml) when the titre was determined on type II cells than on the known model cells for studies on TGEV, ST cells ($10^{5.5}$ per ml). Previous works indicated that the virus in swine testis (ST) cells reaches its maximum titre 72h after infection (unpublished data), while 48h were enough for a higher viral titre in type II cells. Virus titre in type I cells could not be determined as the cells do not show any CPE after TGEV infection.

3.2.7. Detection of TGEV in type II cells using transmission electron microscopy

Next, it was a pleasure for us to be able to see the virus in type II cells under the electron microscope (Fig 34). More interesting was to notice the whole virus at the apical membrane of type II cells, with its typical crown-like morphology. We were also able to observe small transport vesicles carrying the viral particles near big endosome-like (EN) vesicles that also contained viral particles. It was also possible to detect fusion between the viral membrane and the membrane of the vesicles (Fig 34). The virus could not be detected in TGEV infected type I cells.

Detection of TGEV in type II cells using transmission electron microscopy

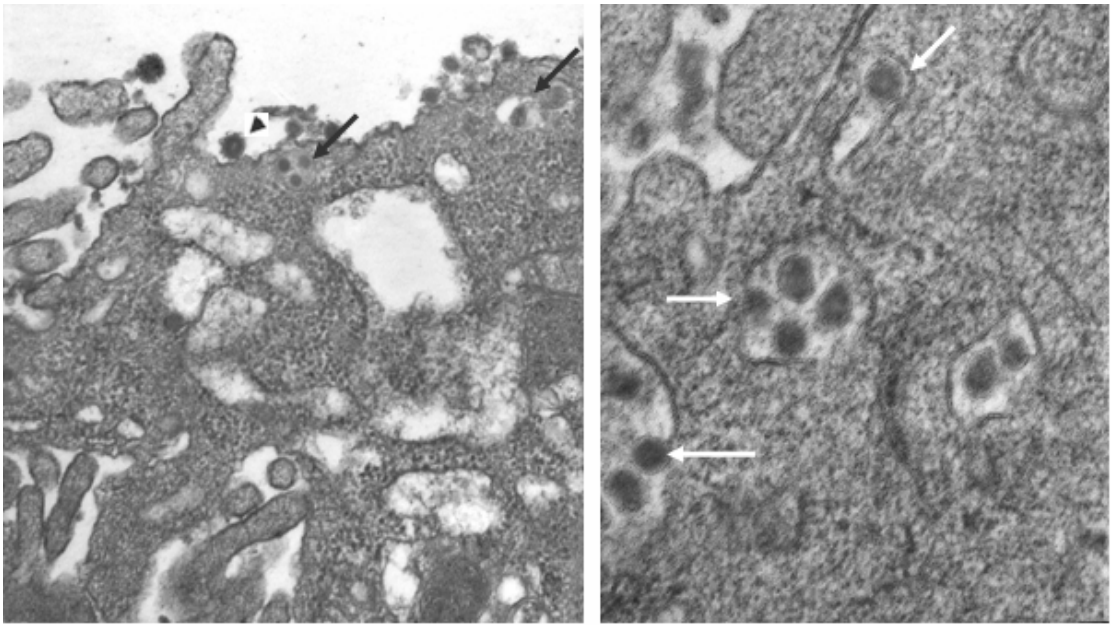


Fig. 34. *TEM showing the* a*pical binding and endocytosis of TGEV by type II cells 24h after infection. A large number of TGEV particles are seen with their typical crown-like morphology (arrow head) in close proximity to the apical membrane and virus particles are also observed in smooth transport vesicles indicated by arrows. TGEV particles are seen with a thickened plasma membrane after adsorption in smooth transport vesicle containing a TGEV particle, which is positioned close to a larger endosome-like vesicle (EN). A continuity between viral and vesicle membranes is also indicated with a white arrow, indicating fusion between the TGEV particle and the intracellular vesicle.*

3.2.8. Antiviral effect of the probiotic pre-treatment on the survival of type II cells after TGEV infection

The data from our MTT (Methylthiazol-diphenyltetrazolium bromide) test showed that treatment of the monolayers of type II cells with either the pellet or filter-sterilized culture-supernatant of the probiotic (containing metabolic products of the probiotic) before infection with TGEV protected the cells from the viral infectivity. Pre-treatment of the monolayers with the pellet of the probiotic resulted in a significant ($P < 0.05$) increase in the survival of both cell types at every dilution (Fig. 35A). Similarly, pre-treatment of the monolayers with all dilutions of the filter-sterilized culture supernatant of the probiotic increased the survival of the infected cells as compared to the non-treated infected monolayers; however the effect was statistically significant ($P < 0.05$) only at the first (2^{-1}) dilution of the supernatant (Fig. 35B).

Increase in the survival of type II cells after TGEV infection as a result of pre-treatment of monolayers with E. faecium SF68

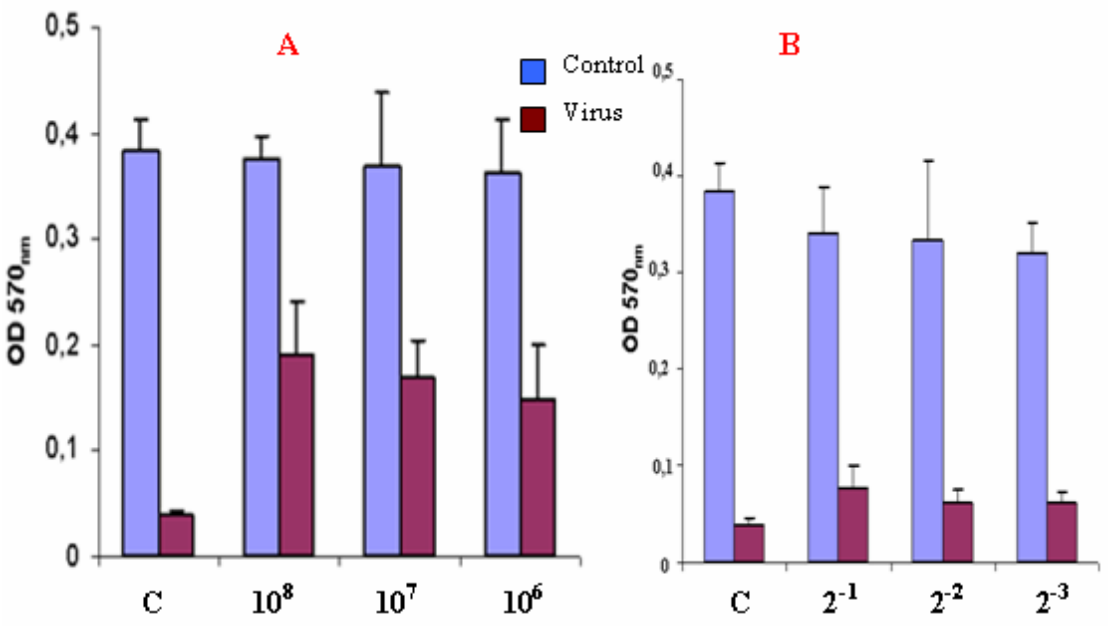


Fig. 35. *Monolayers of type II cells were pre-treated with different dilutions of either the pellet (A) or the filter-sterilized culture supernatant (B) of E. faecium SF68 for 2 h and infected with TGEV as described above. Plates were incubated at 37°C for 72 h and MTT test was undertaken to measure the survival of the cells, which is proportional to the absorbance measured at 570 nm by ELISA plate reader (Tecan). The averages of triplicate measurements are indicated in the graphs. All dilutions of the probiotic pellet, containing 10^8, 10^7 or10^6 bacteria, significantly increased the survival of type II cells as compared to the controls (C)*

that were infected without any pre-treatment. The level of the anti-viral effect proportionally varied with the dilution factors of the pellet or the probiotic pellet. In the case of the antiviral effects associated to the filter-sterilized culture supernatant of the probiotic (B), only the first 1-to-1 dilution (2^{-1}) was statistically significant. The error bars represent the standard deviations of the measurements.

3.2.9. Antiviral effect of the probiotic pre-treatment on virus titre from type II cells after TGEV infection

The increase in the survival of the type II cells after TGEV infection attributed to the probiotic pre-treatment was correlated with a significant decrease in the viral titre. Pre-treatment of type II cells both with the pellet and the culture supernatant of the probiotic resulted in a significant decrease in the virus titre ($TCID_{50}$ml-1) (Fig. 36).

Decrease in the virus titre due to pre-treatment of monolayers with E. faecium SF68

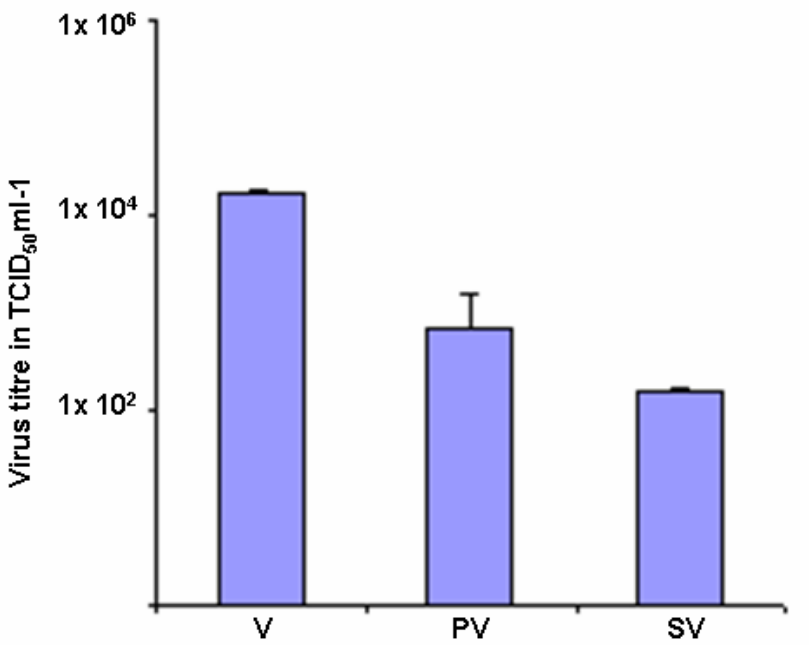


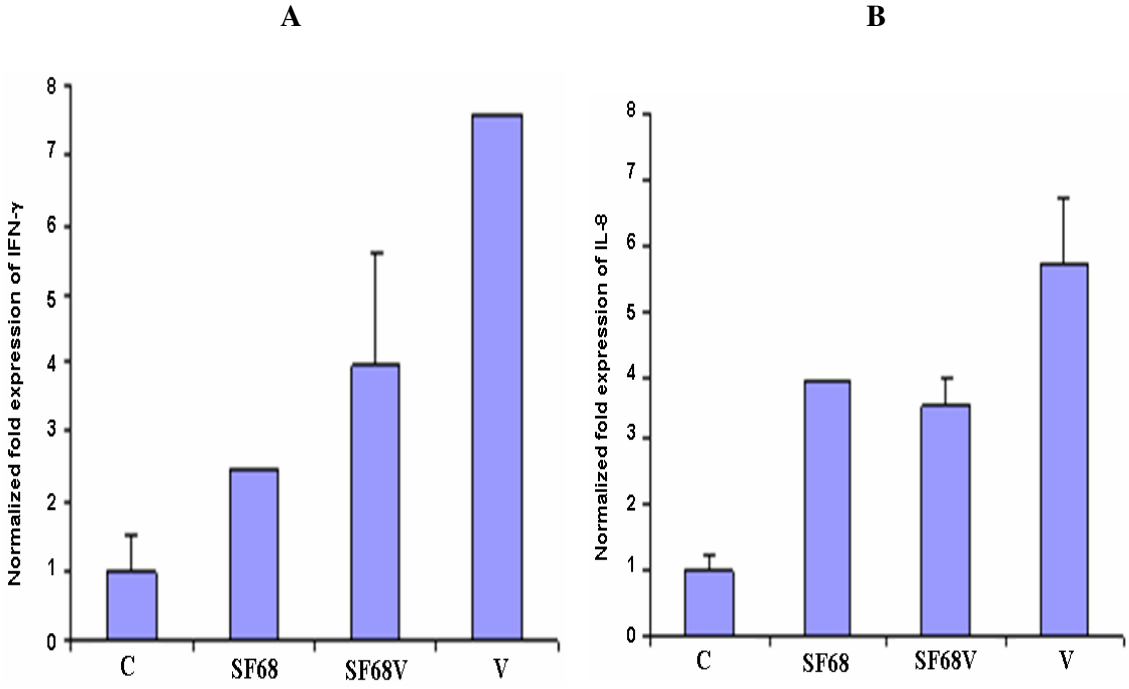
Fig. 36. *Monolayers of type II cells were pre-treated with either the pellet (PV) or the filter-sterilized culture supernatant of E. faecium SF68 (SV) for 2h. Control monolayers were just incubated in DMEM (V). All monolayers were then washed twice and incubated for 1h in DMEM containing 50 µg/ml of gentamicin; after washing, all the cultures were challenged with TGEV ($10^{7.7}$ $TCID_{50}$ ml-1) and further incubated for 2h. Monolayers were washed twice and the medium was replaced with DMEM supplemented with 1% FCS, 1% penicillin/*

streptomycin and 20 µg/ml of gentamicin. Plates were incubated at 37 °C for 72h and virus supernatant as well as intracellular virus was collected by repeated freezing and thawing of the culture plates. The average of the viral titre of triplicate experiments as expressed in $TCID_{50}$ values was significantly ($P < 0.05$) decreased as a result of the pre-treatment either with the probiotic pellet (PV) or its culture supernatant (SV) as compared to the control non-treated virus titres (V). The error bars represent the standard deviations of the measurements of two independent experiments.

3.2.10. Effect of the probiotic pre-treatment on the expression of the cytokines in type II cells

The pre-treatment of the monolayers of type II cells influenced the level of expression of the cytokines IL-6, IL-8 and IFN-γ that were up-regulated by the viral infection. In the case of cells that were pre-treated with the probiotic pellet, the viral-induced up-regulation of the above genes was significantly down-regulated ($P < 0.05$) (Fig. 37). The probiotic pre-treatment alone (SF68) also up-regulated the expressions of the genes to a level that is much lower than that induced by the viral infection (V).

Down-regulation of virus induced expression of cytokines by probiotic pre-treatment



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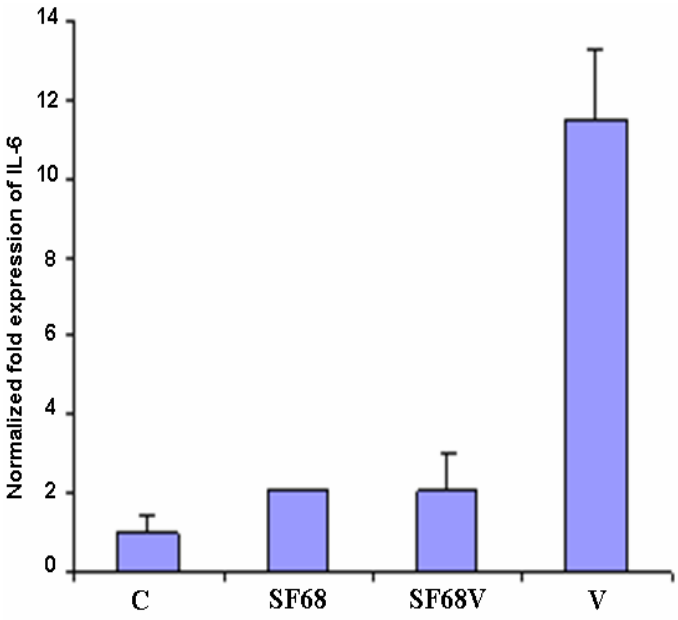

Fig. 37. *Monolayers of type II cells were treated with E. faecium SF68 for 2hrs, washed and incubated in gentamicin containing DMEM. Monolayers were washed and infected with TGEV infection. After 2h, cells were collected for RNA extraction and cDNA synthesis from control cells (C), cell treated only with the probiotic (SF68), cells pre-treated with the probiotic and infected with TGEV (SF68V) and cells infected with TGEV with out probiotic treatment (V). Real-time PCR was performed to compare the expressions of the genes for IFN-γ(A), IL-8 (B) and IL-6 (C). All cytokines were up-regulated by the viral infection (V). The probiotic-pre-treatment alone (SF68) also up-regulated the cytokines to a level much lower than the virus-induced up-regulation. However, the viral-induced up-regulation is down-regulated when the cells are pre-treated with the probiotic (SF68V). The error bars represent the standard deviations of the measurements.*