In vitro effects of the probiotic Enterococcus faecium NCIMB 10415 on adaptive immune cells from German Landrace sows

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Summary

Feeding of the lactic acid-producing Enterococcus faecium NCIMB 10415 (E. faecium), a licensed probiotic for pigs and piglets, has been described to promote growth performance and health in pigs. However, underlying mechanisms of probiotic additives are still elusive as they may directly influence immune cells or influence the intestinal milieu. Here, we established a porcine in vitro cell culture model to explore direct interactions of porcine adaptive immune cells and probiotics. We particularly investigate E. faecium as an alternative dietary additive to improve animals’ health.

To test the direct effects of E. faecium on cytotoxic T-cells and B-cells, two major players of the adaptive immunity, we conducted cell culture experiments with peripheral blood mononuclear cells (PBMCs) of three German Landrace pigs in a co-culture with vital or UV-inactivated E. faecium. For validation, immune cells were repeatedly isolated from blood of three independent donor pigs sharing the same genetic and environmental background. In addition, we analyzed immune cells isolated from mesenteric lymph nodes from adult slaughter pigs. PBMCs were treated with different ratios of PBMCs to E. faecium (1:2, 2:1, 5:1 or 10:1) using different incubation times of 1, 1.5 and 3 hours.

We detected a tendency towards higher relative cell counts of CD8b+ cytotoxic T-cells in the treatment group with vital E. faecium after 1 and 1.5 hours of incubation compared to untreated controls. In addition, we observed a higher activation of cytotoxic T-cells in mesenteric lymph node derived lymphocytes. These results suggest that cytotoxic T-cells are stimulated through vital E. faecium, presumably by secreted factors, whereas inactivated E. faecium had no effect.

By analyzing another important adaptive immune cell type, the B-cells, we observed a different pattern. We found higher relative cell counts of CD21+ B-cells and correspondingly higher relative cell counts of CD79+ B-cells in treatments with UV-inactivated E. faecium, while there was no effect with vital E. faecium, but rather a trend towards lower relative cell counts. In addition, we measured a trend towards lower expression of B-cell regulatory (IGLC, IGKC) and activation marker genes (CD40, CD2) in treatments with vital E. faecium on magnetically sorted CD21+ B-cells.

Hence, we suggest a specific immunomodulatory effect of E. faecium, which presumable influences the direction of immune response towards an enhanced cytotoxic T-cell answer at expense of the B-cell response. This study could provide evidence of a direct immunomodulatory effect of Enterococcus faecium NCIMB 10415 on adaptive immune cells in vitro.

Keywords: pig, probiotics, porcine PBMCs, adaptive immune cells, cytotoxic T-cells, B-cells
Introduction

The time around weaning is considered as the most stressful phase in the life of a piglet (Campbell et al., 2013). Weaning often results in Post-weaning diarrhea (PWD), a serious threat affecting the swine industry worldwide. The disease commonly results in diarrhea, weight loss, and feed failure as a major consequence of weaning (Rhouma et al., 2017).

Since antibiotics and other drugs are banned from farming, alternatives to improve the production, health and welfare of weaned piglets are required. Thus, dietary prebiotics and probiotics have been introduced, which are a major aspect in piglet research. Enterococcus faecium NCIMB 10415 (E. faecium), a widely studied gram positive lactic acid-producing bacterium (LAB) is a licensed probiotic for pigs and piglets in farming since 2005. It has been shown that E. faecium has an immunomodulatory effect in the intestine of pigs in vivo when supplemented as a feed additive (Kreuzer et al., 2012b). However, the underlying direct mechanisms of action of probiotic additives are literally unknown and they are still elusive as they may directly influence immune cells or change the intestinal milieu.

This study aims to enlighten the mechanisms of probiotics, in particular the direct effect of E. faecium on B-cells and cytotoxic T-cells as the major component of the adaptive immune response. The results could provide insights into an alternative dietary additive to improve animals’ health.

Material and methods

For this study, we conducted cell culture experiments with primary cultured porcine immune cells (PBMCs) of three German Landrace pigs in co-culture with either vital or UV-inactivated E. faecium. For validation, immune cells from blood were repeatedly taken from the same animal with the same environmental background. To isolate lymphocytes, 30 ml blood was carefully layered on a 15 ml saccharose-epichlorhydrin-copolymer cushion (Ficoll-Paque™, Sigma-Aldrich Co. LLC). Subsequently, a density gradient centrifugation was performed to separate the mononuclear cells from the residual blood. Mononuclear cells from the interphase were transferred to a PBS-coated (Thermo Fisher Scientific) 50 ml tube and filled with PBS up to 50 ml before centrifugation. After an optional lysis of erythrocytes and another washing step, cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Co. LLC) including glucose, 10 % porcine serum, and 1 % Pen/Strep before cultivation at 37 °C with 5 % CO₂. To isolate B-cells, 1 x 10⁷ cells were separated by Magnetic Activated Cell Sorting (MACS®, Miltenyi Biotec) and cultured under the same conditions as described above. For experiments, either 1 x 10⁶ PBMCs (Tali Image-Based Cytometer, Thermo Fisher Scientific) or 0.5-1 x 10⁶ of separated B-cells were treated with vital or UV-inactivated bacteria (killed via UV-light radiation for 3 hours) from the probiotic strain Enterococcus faecium NCIMB 10415 (Cylaectin, Cerbios Pharma) in a ratio of 1:2, 2:1, 5:1, or 10:1 (PBMCs : E. faecium) for 1, 1.5, 2 or 3 hours in RPMI 1640 without glucose and serum in a 24 well dish (Eppendorf). Thereafter, cells were harvested on ice, centrifuged and washed several times before staining with different dyes for flow cytometry (Table 1). The relative cell count was calculated as the percentage of live lymphocytes. Dead-live discrimination was assessed using DAPI-staining. Otherwise, cells were stored at -80 °C to perform RNA extraction on another day.

Table 1: Dyed cell types, their cell surface marker, and antibodies used.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell surface marker</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
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<tbody>
<tr>
<td>All B-cells</td>
<td>CD79α/ CD79β</td>
<td>CD79α-APC</td>
<td>Directly labeled</td>
</tr>
<tr>
<td>Mature B-cells</td>
<td>CD21</td>
<td>CD21-FITC</td>
<td>Directly labeled</td>
</tr>
<tr>
<td>Cytotoxic T-cells</td>
<td>CD8α/ CD8β</td>
<td>CD8β</td>
<td>Anti-mouse IgG2a PE</td>
</tr>
<tr>
<td>Activated Cells</td>
<td>CD27</td>
<td>CD27</td>
<td>Anti-mouse IgG1 APC</td>
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</tbody>
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For expression analysis, RNA was extracted by a NucleoSpin® RNA isolation Kit (MACHEREY-NAGEL), transcribed into cDNA using SuperScript® VILO™ (Invitrogen), and analyzed by qPCR (ViiA 7, Thermo Fisher Scientific) using Brilliant III Ultra-Fast SYBR® Green Master-Mix (Agilent Technologies). The expression of B-cell specific genes Immunoglobulin kappa constant (IGKC), Immunoglobulin lambda light C region (IGLC), cluster of differentiation 2 (CD2), cluster of differentiation 40 (CD40) was studied. RPL19 was used as a housekeeping gene. CD2 (forward 5’ AGTAACAACGCAGTGAGCA 3’ and reverse 5’ TCTCCTGCTGCTCTGCTTTT 3’) and CD40 (forward 5’ CCAGTTGGCTTCTTCTCCA 3’ and reverse 5’ AACAGGACGGCAAACAGGAT 3’) were amplified at an annealing temperature of 60°C with self-designed primer pairs using Primer3. Primer pairs for IGLC, IGKC, and RPL19 were used as described earlier (Kreuzer-Redmer et al., 2016). The colony forming unit of the bacteria as well as the “killing”-effect of the UV-light were examined by smear from culture on Columbia Agar plates (Sigma-Aldrich Co. LLC). Significance between different treatments was assessed using Student’s t-test for gene expression levels and using Mann-Whitney-U-test for relative cell counts. Significant p-values are illustrated in each figure with “+” for p < 0.1 and “*” for p < 0.05.

**Results**

To prove the hypothesis that *E. faecium* influences the immune status of pigs via a direct interaction of *E. faecium* and immune cells, we conducted in vitro experiments with primary cultured porcine immune cells.

We detected a tendency towards higher relative cell counts of CD8b+ cytotoxic T-cells (p < 0.1) in the treatment group with vital *E. faecium* after 1 and 1.5 hours of incubation compared to untreated controls within PBMCs from three German Landrace pigs (as biological replicates) in two independent experiments (Figure 1A). In a separate experiment, in which we treated primary cultured lymphocytes derived from two slaughter pig’s mesenteric lymph nodes with *E. faecium*, we co-stained CD8b+ cells with the activation marker CD27. If CD27 is lost, T-cells had antigen contact and were therefore activated (Reutner et al., 2012). We found a tendency towards higher relative cell counts of CD8+CD27- cytotoxic T-cells for lymphocytes treated with vital *E. faecium* bacteria compared to the control with no *E. faecium* treatment (Figure 1B). Treatment with UV-inactivated bacteria did not result in changes of the relative cell count of cytotoxic T-cells.
Figure 1. In vitro effects of E. faecium on PBMCs (A) and on primary cultured lymphocytes isolated from mesenteric lymph nodes (mLN) from two adult slaughter pigs (B). Cell counts of CD8b+ cytotoxic T-cells as well as of CD8b+CD27+ and CD8+CD27- (activated) cytotoxic T-cells are relative to the living (DAPI negative cells) lymphocyte population obtained by flow cytometry. Cells were not treated (control) or treated with either vital Enterococcus faecium NCIMB 10415 (EF) or UV-inactivated EF in a ratio of 10 PBMCs to 1 EF, 5 PBMCs to 1 EF and 2 PBMCs to 1 EF for 1.5 hours.

Furthermore, we were interested in analyzing the interaction of E. faecium treatment to another important adaptive immune cell type, the B-cells. After 1.5 hours of incubation of PBMCs from three German Landrace pigs (as biological replicates) in three independent experiments with UV-inactivated E. faecium, we measured higher relative cell counts of CD21+ B-cells (P < 0.05) and correspondingly we detected a tendency towards higher relative cell counts of CD79+ B-cells (P < 0.1) than in controls, while there was no effect with vital E. faecium, but rather a trend towards lower relative cell counts with vital E. faecium bacteria on CD21+ and CD79+ B-cells (Figure 2).

Figure 2. In vitro effects of E. faecium on PBMCs. Cell counts of CD21+ and CD79+ B-cells are relative to the live (DAPI negative cells) lymphocyte population obtained by flow
cytometry. Cells were not treated (control) or treated with either vital Enterococcus faecium NCIMB 10415 (EF) or UV-inactivated EF in a ratio of 10 PBMCs to 1 bacterium, 5 PBMCs to 1 bacterium and 2 PBMCs to 1 bacterium for 1.5 hours.

To further examine B-cell activation, we measured gene expression in magnetically separated CD21+ B-cells, which were not treated or treated with vital E. faecium. In a first pre-experiment with blood from two pigs, we found a trend towards a lower expression of the B-cell regulatory genes IGLC and IGKC, as well as for the activation marker genes CD40 and CD2 in mesenteric lymph nodes and PBMCs treated with vital E. faecium than in untreated controls after 1.5 hours of incubation (Figure 3).

![Figure 3. In vitro effects of E. faecium on sorted B-cells. CD21+ B-cells were separated by Magnetic Cell Sorting (Miltenyi), and treated (green bars) or not treated (blue bars) with vital E. faecium bacteria.](image_url)

**Discussion**

Probiotics are meaningful dietary additives, which could improve the health condition of pigs after weaning. However, mechanisms of the interaction of probiotics with cells of the adaptive immune system like B-cells and T-cells remain elusive. Therefore, we established a porcine in vitro cell culture model to explore the direct interactions of porcine adaptive immune cells and probiotics.

We detected higher relative cell counts of CD8b+ cytotoxic T-cells with treatment of vital E. faecium bacteria within PBMCs. Additionally, we observed a higher activation of cytotoxic T-cells within lymphocytes derived from mesenteric lymph nodes, which were co-cultured with vital E. faecium. That hints towards a stimulation of cytotoxic T-cells through vital E. faecium. These results suggest that cytotoxic T-cells need a vital bacterium and were presumably stimulated by secreted substances of E. faecium. Further experiments will be done to uncover the role of secreted factors of E. faecium in activation of cytotoxic T-cells.

By analyzing another important adaptive immune cell type, the B-cells, we observed a different pattern. We found higher relative cell counts of CD21+ B-cells and correspondingly higher relative cell counts of CD79+ B-cells in treatments with UV-inactivated E. faecium, while there was no effect with vital E. faecium, but rather a trend towards lower relative cell counts.
counts with vital *E. faecium* bacteria. In addition, we measured lower expression of B-cell regulatory and activation marker genes in treatments with vital *E. faecium*. Lower expression of IGLC and IGKC, encoding the constant part of immunoglobulins and thus being important for B-cell regulation was described in ileal lymph nodes from *E. faecium* fed piglets (Kreuzer-Redmer et al., 2016). The obtained *in vitro* data from this study shows the same direction of effect as the former *in vivo* study.

Thus, we suggest, that *E. faecium* influences the direction of immune response towards an enhanced response of cytotoxic T-cells at the expense of B-cells, which are rather reduced. This is also reflected by data of former animal trails. Cytotoxic T-cells are important to control virus infections. We found reduced Rotavirus A and no Astrovirus shedding in *E. faecium* fed piglets and mother sows in relation to controls with no *E. faecium* feeding (Kreuzer et al., 2012b). In contrast, we did detected higher loads of *Salmonella Typhimurium* in *E. faecium* fed weaned piglets in another *in vivo* study (Kreuzer et al., 2012a). To control *Salmonella Typhimurium*, B-cells are an important player within the adaptive immunity. This hints towards a specific immunomodulatory effect of *E. faecium* on adaptive immune cells.

This ongoing study could provide evidence of a direct immunomodulatory effect of *Enterococcus faecium* NCIMB 10415 on adaptive immune cells *in vitro*.

**List of References**


