Effects of *Enterococcus faecium* NCIMB 10415 on porcine immune cells

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Background

➢ Improvement of production, health and welfare of weaned piglets
➢ Reduction of antibiotics and other drugs
➢ Feeding of probiotics as alternative

➔ What are the mechanisms of action of the probiotics?
Background

*Enterococcus faecium* NCIMB 10415 (EF):

- Gram positive, lactic-acid producing bacterium
- Licensed probiotic for pigs since 2005 in Germany
- Pharmaceutical probiotic in humans: „Bioflorin®“ , „Newflora™“

Known effects in swine:

- Reduction of incidence and severity of diarrhea (Busing and Zeyner, 2015; Taras et al., 2006; Zeyner and Boldt, 2006)
- Reduced the number of mucosa-adherent *Escherichia Coli* pathotypes (Bednorz et al., 2013)
- Modulation of the intestinal immune system (Kreuzer et al., 2012; Scharek et al., 2005; Wang et al., 2014)
Background – Adaptive immune cells
Background – *in vivo* EF effects on immune cells in feeding experiments

*E. faecium*-treatment increased the relative cell count of cytotoxic T- and B-cells preweaning in peripheral blood mononuclear cells.

Kreuzer et al. *Veterinary Research* 2012, 43:58
Background – Hypothesis

➢ *E. faecium* is able to directly affect adaptive immune cells.

➢ *E. faecium* activates cytotoxic T cells and B-cells

1. Does *E. faecium* directly effect the immune system?

2. Which component of *E. faecium* mediates the immunomodulatory effects?
Materials & Methods - Experimental Design

Probiotic strain used: *Enterococcus faecium* NCIMB 10415/SF68
Non-probiotic strains used: *E. faecium* 2918, *E. faecium* 20477

German Landrace pigs

Mesenteric lymph nodes (Slaughter pigs)

Isolated immune cells (PBMCs, LNLs)

Secreted factors

Separated B- and T-cells

B- and T-cells were analyzed on cell and transcript level by flow cytometry and qPCR
In vitro effects of EF on cytotoxic T-cells in PBMCs

- Tendency towards higher relative cell count of cytotoxic T-cells with vital *E. faecium* treatment suggests an involvement of secreted factors.
**In vitro** effects of EF on **cytotoxic T-cells** in LNLs

- **Naive T-cells**
  - CD8β⁺ CD27⁺

- **Activated T-cells**
  - CD8β⁺ CD27⁻

- Tendency towards a higher relative cell count of activated cytotoxic T-cells with vital *E. faecium* treatment suggests involvement of secreted factors.
In vitro effects of different EF strains on cytotoxic T-cells in PBMCs

Higher variation in immune response

- Higher relative cell count of cytotoxic T-cells with treatment with the probiotic E. faecium strain suggests a strain-specific, probiotic effect
In vitro effects of EF on B-cells in PBMCs

- Higher relative cell count of B-cells with killed *E. faecium* treatment suggests an involvement of a surface compound.
- Lower relative cell count of B-cells with vital *E. faecium* suggests an inhibition by secreted factors.
In vitro effects of different EF strains on B-cells in PBMCs

- Lower relative cell count of B-cells with vital *E. faecium* suggests a strain-specific, probiotic effect
**In vitro** effects of EF on sorted B-cells

Higher relative cell count of primed B-cells with vital and killed *E. faecium* treatment suggests a different mode of action on sorted B cells than in a PBMC or LNL composite.
Summary & conclusion

- **Vital** *E. faecium* seemed to inhibit B-cells and increased cytotoxic T-cells in PBMCs and LNLs composite, which could be mediated through secreted factors of *E. faecium*

- **Killed** *E. faecium* increased B-cells in PBMCs which might suggest an involvement of a surface compound

- The effects of *E. faecium* NCIMB 10415 seem to be strain-specific

- **Vital and killed** *E. faecium* increased primed B-cells in sorted B-cells which might suggest activation via bacterial components as secreted factors or surface molecules

- There is evidence of a direct immunomodulatory effect of *Enterococcus faecium* NCIMB 10415 on adaptive immune cells
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THANK YOU FOR YOUR ATTENTION!
Pre-experiments

Testing the cfu (colony forming unit) of the used product (Cylactin, Cerbios Pharma)

Columbia-Agar plates incubated with EF
Pre-experiments

• Testing the “killing” effect of UV to *Enterococcus faecium*

UV-light for 60 min
UV-light for 40 min
UV-light for 20 min
live
Pre-experiments

- Testing the “killing” effect of UV to *Enterococcus faecium*

Columbia-Agar plates incubated with EF
Key findings


➢ Characterization of CD4+ subpopulations and CD25+ cells in ileal lymphatic tissue of weaned piglets infected with *Salmonella Typhimurium* with or without *Enterococcus faecium* feeding (Kreuzer et al. *Vet Immunol Immunopathol* 2014, 158:143-155).


In vitro effects of EF on cytotoxic T-cells in a PBMC and LNL composite

- Tendency towards higher relative cell counts of cytotoxic T-cells (CD8β+) with vital *E. faecium*

- Tendency towards a higher relative cell counts of activated cytotoxic T-cells (CD8β+ CD27+) with vital *E. faecium* bacteria in mesenteric lymph nodes (mLN)
Fluorescence Intensity of CD21-APC

MACS (-)

CD21+ 4.08

S47_MACS_CD21intfluos.txt
Line 34970

Fluorescence Intensity of CD21-APC

MACS (+)

CD21+ 89.1

S47_MACS_CD2plus_fcs.txt
Line 16555

Fluorescence Intensity of CD8-PE

CD21+, CD8b+ 15.2

Q2 0.35

G4 81.6

CD21+, CD8b- 2.92

S47_PluriCD21plus_001.fcs
Line 568

Fluorescence Intensity of CD21-APC

PluriSelect (+)

CD21+ 41.9

S47_PluriCD21plus_001.fcs
Line 568

CD21+, CD8b+ 0.70

Q2 0.35

G4 67.9

CD21+, CD8b- 41.0

Cellsort – (pre)experiments
In vitro effects of different EF strains on cytotoxic T-cells in a PBMC composite

Problem: High variation in immune response capacity between animals and within one animal across the seasons !!!

Relative cell count (%)

CD8β+

3 h EF-Treatment Ratio PBMC : EF

Control

2:1 vEF-SF68

2:1 vEF-20477

2:1 vEF-2918

Relative cell count (%)

Δ Relative cell count (%)

3 h EF-Treatment Ratio PBMC : EF

Pig

S47-a

S47-b

S47-c

S51-a

S51-b

S51-c

S52-a

S52-b

S52-c

S54-a

S54-b

S54-c
**In vitro** effects of EF on cytotoxic T-cells in a PBMC composite

Higher relative cell counts of cytotoxic T-cells after 5 h incubation with *E. faecium*
In vitro effects of EF on B-cells in a PBMC composite

- Trend to lower relative cell counts of B-cells (CD21, CD79) with vital E. faecium
- Higher relative cell counts of B-cells (CD21, CD79) in killed E. faecium
*In vitro* effects of different EF strains on **B-cells** in a PBMC composite

Lower relative cell counts of **B-cells** with vital *E. faecium* SF68.
**In vitro** effects of EF on **separated cytotoxic T-cells**

No clear effect on **activated and naive cytotoxic T-cells** after treatment with vital *E. faecium*
Flow cytometry – Gating strategy
T-cell-independent B-cell activation

TI-1 antigens:
- directly cause proliferation and differentiation (TLR) (polyclonal when high concentration or antigen-specific)
- BCR-crosslink or other forms of costimulation (LPS – LPS-receptor)
- B-cell mitogens
- Examples: LPS, bacterial DNA

TI-2 antigens:
- Highly repetitive surface structures
- Cross-linking of BCRs leading to cross-activation
- Need residual T-cell, DC or MP help for activation (costimulatory signals)

Introduction - CD79

- Encompasses two transmembrane proteins
- Parts of BCR
  - Signal transduction
- First components of BCR expressed developmentally

Woyach, J. A. et al. (2012)
Introduction - CD2

• Cell adhesion molecule on T- and NK-cells

• Four different subsets

<table>
<thead>
<tr>
<th>Subsets</th>
<th>Function</th>
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<tbody>
<tr>
<td>CD2⁺CD21⁺</td>
<td>Mainly naive B-cells</td>
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<tr>
<td>CD2⁻CD21⁺</td>
<td>Primed B-cells</td>
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<tr>
<td>CD2⁺CD21⁻</td>
<td>Active antibody forming &amp; plasma cells</td>
</tr>
<tr>
<td>CD2⁻CD21⁻</td>
<td>Resting antibody forming &amp; plasma cells</td>
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– Developmental marker
Introduction - CD21

- **CD21:**
  - Complement receptor type 2 (CR2)
  - All mature B lymphocytes
  - Allows B-cell activation by complement
  - Maturation marker
  - Two differential forms
Results - MACS-efficacy

MACS\textsuperscript{®} (-)  
MACS\textsuperscript{®} (+)

Forward scattered light

CD21-FITC fluorescence

CD21\textsuperscript{+}

96,0 \%

Good B-cell sort-efficacy!