Supplementations of *Bacillus* spp. on gut microbiota of weaned pigs under diarrheal stress

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• The digestive system (gut) is crucial for nutrient metabolism and immunity regulation in pigs

• Microbes are to harbor innumerable in the gut and have been overlooked to understand their benefits for the pig (host)

• The dynamic and diverse population of microbes in the gut is collectively referred to as the **gut microbiota**
Microbes and their host hold a **symbiotic relationship**, in which the host allows microbes to habituate in the intestines while the microbes benefits the host’s health.
• **Weaning** becomes one of the most stressful moments in pigs
  • Separation from sow and possibly their littermates
  • Change of environment
  • Diet change

• **Weaning stress** reduces feed intake, which can cause a cascading effect toward pig’s health
  • Lower feed intake = less metabolizable energy = disturbance of intestinal development in growing pigs
Post-weaning diarrhea

• Post-weaning stress can cause diarrhea
• Post-weaning diarrhea is commonly induced by enterotoxigenic *E. coli* (ETEC)
• Leads to high morbidity rate in weaning pigs along with huge economical loss in the swine industry


Bacillus spp.: Potential feed additives

• Our previous study (He et al., 2020) observed that supplementing *B. subtilis* reduced diarrhea and enhanced growth performance of weaned pigs experimentally infected with ETEC.
Bacillus spp.: Potential feed additives

However, impact of Bacillus spp. on gut microbiota of weaned pigs under post-weaning diarrhea is not yet understood

Objective 1

Supplementations of Bacillus (B.) subtilis on intestinal microbiota on weaned pigs challenged with ETEC

Objective 2

B. amyloliquefaciens (BAM) supplementation on performance, systemic immunity, and intestinal microbiota of weaned pigs challenged with ETEC
Supplementations of *B. subtilis* on intestinal microbiota on weaned pigs challenged with ETEC

Objective 1
Materials & methods

- 48 weaned pigs (6.17 ± 0.36 kg)
  - Around 21-24 day of age
- 4 treatments (12 pigs / treatment)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
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<tbody>
<tr>
<td>Sham</td>
<td>NC</td>
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<tr>
<td></td>
<td>Negative control with basal diet</td>
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<tr>
<td>ETEC</td>
<td>PC</td>
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<tr>
<td></td>
<td>Positive control with basal diet</td>
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<tr>
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<td>AGP</td>
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<td></td>
<td>Basal diet with 50 mg/kg carbadox</td>
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<tr>
<td></td>
<td>DFM</td>
</tr>
<tr>
<td></td>
<td>Basal diet with 500 mg/kg <em>B. subtilis</em></td>
</tr>
</tbody>
</table>

AGP = antibiotics growth promoter (carbadox)
DFM = Direct fed microbials (*B. subtilis*)
ETEC inoculation
$10^{10}$ CFU per 3-mL dose in PBS

- Pigs were screened for ETEC sensitivity prior to experimentation prior to study
- *Ad libitum* water and feed provided throughout the study

PI = Post Inoculation
Sample collection

- Fecal samples collected
- Jejunal digesta, ileal digesta, and cecal content

Allotment

- d -7
- 0
- 1
- 2
- 7

Necropsy

21 PI
16S rRNA sequencing

1. Library prep
   - DNA extraction
   - PCR
   - Illumina sequencing

2. Downstream analysis
   - sabre
   - QIIME2

3. Data analysis
   - R program
   - Statistical analysis
   - Data visualization
Gut microbiota analysis

**Alpha diversity:**
- Shannon index (richness & evenness)
- Chao1 index (richness only)

**Beta diversity:**
- Bray-Curtis dissimilarity

**Taxonomic analysis:**
- Relative abundance in phylum and genus levels
Statistical analysis

- **Alpha diversity and relative abundance**
  - Normal distribution = ANOVA followed by estimated marginal means (EMMEANS)
  - Non-normal distribution = Kruskal-Wallis followed by Conover test

- **Beta diversity**
  - Betadisper followed by adonis function using vegan package in R
Results: Fecal microbiota

Alpha diversity: Chao1 index (richness only)

- Chao 1 index was increased between d -7 and d 0 for all dietary treatments

No significant difference observed in Shannon diversity (richness & evenness)

NC = no ETEC
PC = w/ ETEC
AGP = carbadox
DFM = B. subtilis

\[ P < 0.05 \]
Feces: Beta diversity

- Each point represents a sample.
- Color and shape represent the day sampled.
- The farther the point is from another point, the more likely there is a difference in microbial composition between the two points.
Feces: Beta diversity

Treatment*Day interaction

- Facetted by sampling days
- Color and shape represent treatment
Feces: Phylum

Relative abundance: Phylum level

- **Firmicutes** increased over time
- **Bacteroidetes** decreased over time
- **Proteobacteria** remained the same
**Feces: Phylum**

**Relative abundance: Phylum level**

- **Firmicutes** increased over time
- **Bacteroidetes** decreased over time
- **Proteobacteria** remained the same

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**Graphs**

- **Firmicutes**: Relative abundance over time from d-7 to d21, marked with an asterisk indicating a significant change.
- **Bacteroidetes**: Decrease over time, marked with an asterisk.
- **Proteobacteria**: Showing no significant change over time, marked with an asterisk.
Feces: Genus

- *Lactobacillus* was greater in DFM than in AGP on d 0 and 21 PI

Relative abundance: Genus level
Results: Intestines

Alpha diversity: Intestinal sites

- Samples were collected on d 21 PI
- Shannon and Chao1 indices were greatest in colon
Beta diversity: Intestinal sites

Color and shape represent intestinal site (jejunum, ileum, or colon)
Intestines: Beta diversity

Beta diversity: Site*Treatment

Color and shape represent treatment
Intestines: Phylum

Relative abundance: Phylum level

• **Actinobacteria** was greater in ileal digesta from PC than from AGP
• **Bacteroidetes** was greater in AGP than in DFM in ileal digesta
• **Proteobacteria** was greater in AGP than in DFM in jejunal and ileal digesta
Intestines: Genus

Relative abundance: Genus level

• *Lactobacillus* and *Bifidobacterium* were greater in DFM than AGP and PC in jejunum, ileum, and colon on d 21 PI
Conclusion

• Age, intestinal sites, ETEC, and diet all contributed to the modulation of gut microbiota

• **Carbadox** supplementation increased relative abundance of gram-negative bacteria including Bacteroidetes and Proteobacteria
  • Could increase risk of antibiotics resistance

• **B. subtilis** supplementation was associated with increase of beneficial microbes in the intestinal microbiota of weaned pigs under ETEC challenge
B. amyloliquefaciens (BAM) supplementation on performance, systemic immunity, and intestinal microbiota of weaned pigs challenged with ETEC

Objective 2
Materials & methods

- 50 weaned pigs (7.41 ± 1.35 kg)
  - Around 21-24 day of age
- 5 treatments (10 pigs / treatment)

<table>
<thead>
<tr>
<th>Sham (-)</th>
<th>CON -</th>
<th>Control diet</th>
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<tbody>
<tr>
<td></td>
<td>BAM -</td>
<td>0.10% inclusion rate with 10^9 CFU/kg BAM</td>
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<tr>
<td>ETEC (+)</td>
<td>CON +</td>
<td>Control diet</td>
</tr>
<tr>
<td></td>
<td>BAM +</td>
<td>0.10% inclusion rate with 10^9 CFU/kg BAM</td>
</tr>
<tr>
<td></td>
<td>AGP +</td>
<td>50 mg/kg of Carbadox</td>
</tr>
</tbody>
</table>
ETEC inoculation
$10^{10}$ CFU per 3-mL dose in PBS

- Pigs were screened for ETEC sensitivity prior to experimentation
- *Ad libitum* water and feed provided
Measurements

1. Growth performance
   • Bodyweight
   • Average daily gain (ADG)
   • Average daily feed intake (ADFI)

2. Diarrhea frequency
   • Diarrhea score (1 = normal feces, 5 = watery diarrhea)

3. Systemic immunity
   • Total and differential blood cell count

4. Fecal and ileal microbiota
   • Alpha and beta diversity
   • Relative abundance
Statistical analysis

- **PROC MIXED of SAS**
  - Randomized complete block design
  - Pig as experimental unit
  - Diet and challenge as main effect
  - Block as random effect
Statistical analysis: Gut microbiota

• Alpha diversity and relative abundance
  • Normal distribution = ANOVA followed by estimated marginal means (EMMEANS)
  • Non-normal distribution = Kruskal-Wallis followed by Conover test

• Beta diversity
  • Betadisper followed by adonis function using vegan package in R
### Results

#### Bodyweight

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SHAM (-)</th>
<th>ETEC (+)</th>
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<tbody>
<tr>
<td></td>
<td>CON</td>
<td>BAM</td>
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<tr>
<td>d 21 PI</td>
<td>$17.92^{ab}$</td>
<td>$18.09^a$</td>
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</table>

$P < 0.05$

**Legend:**
- **CON-** = no ETEC
- **BAM-**
- **CON+** = w/ ETEC
- **BAM+**
- **AGP** = carbadox
Growth performance

$P < 0.05$
**Frequency of diarrhea**

DS = Diarrhea score

DS 1 = normal feces

DS 5 = watery diarrhea

% of subjects with DS ≥ 3 and DS ≥ 4 are shown in the bar chart. The chart indicates the percentage of subjects in different groups (CON-, BAM-, CON+, BAM+, AGP+) with DS values of 3 and 4.

- **DS ≥ 3**: Bars labeled with different letters (a, ab, c) indicate significant differences among groups.
- **DS ≥ 4**: Similar letter labeling for significant differences.

*P < 0.05*
# WBC counts

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<th>Day PI</th>
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<th>ETEC (+)</th>
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<td>CON</td>
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**Lymphocyte, 10^3/µL**

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<td>CON</td>
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- **Day 0**: SHAM - CON: 4.86b, BAM: 4.75b; ETEC - CON: 5.23b, BAM: 6.31ab, AGP: 7.14a
- **Day 7**: SHAM - CON: 5.28b, BAM: 6.21ab; ETEC - CON: 8.71a, BAM: 8.26a, AGP: 8.22a
- **Day 21**: SHAM - CON: 5.59b, BAM: 5.63b; ETEC - CON: 7.30a, BAM: 5.36b, AGP: 6.79ab

**Neutrophil, 10^3/µL**

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- **Day 14**: SHAM - CON: 7.62b, BAM: 7.22b; ETEC - CON: 9.49a, BAM: 7.92b, AGP: 7.27b
- **Day 24**: SHAM - CON: 5.45ab, BAM: 5.46ab; ETEC - CON: 5.96a, BAM: 4.40bc, AGP: 4.67c

**P < 0.05**
Fecal microbiota

Beta diversity

Color and shape represent sampling day
Feces: Beta diversity

Treatment*Day
- AGP+ samples were clustered together
Feces: Phylum

Firmicutes decreased between d 0 and 7 PI in AGP+
Proteobacteria increased between d 0 and 7 PI in CON and AGP
Feces: Genus

- *Lactobacillus* was greater in CON+ than CON- on d 7 PI
- *Blautia* and *Prevotella* was greater in AGP+ than in BAM+ on d 0
- *Prevotella* was greater in BAM+ than AGP+ in d 21 PI
Ileal microbiota

Alpha diversity (Shannon)

- Ileal digesta collected on d 21 PI
- CON+ and BAM+ had greater Shannon diversity index than AGP+ in ileum
Ileum: Beta diversity

Separated clusters in ETEC infected and non-infected treatment groups in ileal digesta.
Ileum: Phylum

- **Firmicutes** was greater in AGP+ than in CON+ and BAM+ in ileal digesta.
- **Proteobacteria** was greater in CON+ than in CON-.
• **Lactobacillus** was greater in CON- than in ETEC infected groups
• **Clostridium sensu stricto** 1 was more abundant in AGP+ than BAM+ in ileal digesta
Conclusions

• *B. amyloliquefaciens* supplementation to weaned pigs challenged with ETEC
  • Tended to enhance growth performance
  • Had limited effects on diarrhea
  • Reduced systemic inflammation
  • Altered fecal and ileal microbiota differently from carbadox supplementation

• *B. amyloliquefaciens* solely may not provide weaned pigs with growth enhancement and acute diarrheal alleviation as similarly as carbadox

*Bacillus* spp. can vary in how they are used how they impact different animal species
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