Anaerobic Fermentation to Produce Carboxylic Acids and Inactivate *Ascaris* Eggs

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Our Goal:
Inactivate pathogens in fecal solids through *in-situ* production of carboxylic acids
Outline

Introduction
• What are carboxylic acids?
• Carboxylic acids and pathogens
• Production of carboxylic acids

Methods and Results
• Batch fermentation
• Inactivation of *Ascaris* eggs

Summary and Future Work
What is a carboxylic acid?

**Carboxylic acid**: a weak organic acid containing a carboxyl group

\[
\begin{align*}
\text{O} & \\
\text{C} & \\
\text{R} & \\
\text{OH}
\end{align*}
\]

We most often think of **fatty acids** (hydrocarbon chains followed by a carboxyl group).

- **Acetic acid**  \(\text{C}2\)
- **\(n\)-Butyric acid**  \(\text{C}4\)
- **\(n\)-Caproic acid**  \(\text{C}6\) (Hexanoic Acid)
A note about weak acids

\[ n\text{-Butyric Acid} \rightleftharpoons K_a \quad n\text{-Butyrate} + H^+ \]

\[ \text{pH} = \text{p}K_a + \log \left( \frac{[A^-]}{[HA]} \right) \]

\( pK_a = 4.8 \)
Carboxylic acids and pathogens

1. Membrane disruption
2. Decrease internal pH
3. Anion accumulation
4. ATP used to pump out protons
Ascaris as a model pathogen

Carboxylic acid production and chain elongation

Organic Waste → Hydrolysis → Monomers

- Hydrolysis
- Monomers

- Fermentation
  - Short-Chain Carboxylic Acids (e.g., Acetic Acid)

- Methanogenesis
  - Methane

H₂ + CO₂

Diagram:
- Organic Waste
- Hydrolysis
- Monomers
- Fermentation
- Short-Chain Carboxylic Acids (e.g., Acetic Acid)
- Methanogenesis
- Methane
Chain Elongation

- Longer Chain Carboxylic Acids (e.g., n-Butyric Acid)
- Short-Chain Carboxylic Acids (e.g., Acetic Acid)

Reduced Substrate

Acetyl-CoA
Chain Elongation

Short-Chain Carboxylic Acids (e.g., n-Butyric Acid)

Longer Chain Carboxylic Acids (e.g., n-Caproic Acid)

Reduced Substrate

Acetyl-CoA
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Summary and Future Work
Batch fermentation to produce carboxylic acids

- Fecal solids and inoculum from a carboxylate-producing reactor
- Incubated anaerobically at 30°C
- Sampled every 4-7 days for 69 days
Batch fermentation to produce carboxylic acids

- **Max n-butyric acid**: 257 mM
- **Max n-caproic acid**: 27 mM
- **Average pH**: 6.2 (SD=0.6)
Parameters controlling *Ascaris* viability

- Concentration
- pH
- Carbon chain length
- Exposure time
- Temperature
**Ascaris viability: Carboxylic acid concentration and pH**

- Three pH levels tested (pH= 2,4,5)
- Same concentrations of uncharged acids (HA) tested at each pH

\[
\text{pH} = \text{pK}_a + \log \left( \frac{[A]}{[HA]} \right)
\]

\[
[HA + A^-] = [HA] \left(10^{(\text{pH} - \text{pK}_a)} + 1\right)
\]
Ascaris viability: Carboxylic acid concentration and pH

- Exposed for 3 days at 37°C

- Washed and incubated for 21 days at 30°C

- 500 eggs examined for viability

Harroff, 2015
Ascaris viability not dependent on anion concentration or pH

\[ y = \frac{1}{1 + \exp\left(-a(x - b)\right)} \]

Sum of Uncharged \( n \)-Butyric and \( n \)-Caproic Acid Concentrations (mM)
Parameters controlling *Ascaris* viability

- **Concentration**
- **pH**
- **Carbon chain length**
- **Exposure time**
- **Temperature**
Ascaris viability: Concentration, chain length, and exposure time

- *A. suum* eggs exposed to *n*-butyric acid and *n*-caproic acid individually

- pH 2

- Concentration and exposure time varied

- T=30°C

- Anaerobic
Ascaris viability dependent on concentration, chain length, and exposure time

\[ y = \frac{1}{1 + \exp(-a(x - b))} \]
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Summary and Future Work
Conclusions

- We can produce carboxylic acids from fecal solids.
- The concentration of uncharged acid is important.
- For individual acids, we can predict *Ascaris* inactivation based on exposure time and concentration.
Future Work

• Decrease pH through fermentation

• Understand different parameters relating to inactivation:
  • Temperature
  • Mixtures of acids
  • Matrix

• Scale up
Acknowledgements

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*Ascaris* inactivation
- Dr. Michael P. Labare, PhD
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- Dr. Nzuhat Islam, MS, DVM
- Zhu Dan, MEng

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- Dr. Lynn Johnson, PhD
  Cornell Statistical Consulting Unit

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- National Science Foundation Graduate Research Fellowship Program
Conclusions

• We can produce carboxylic acids from fecal solids.

• The concentration of uncharged acid is important.

• For individual acids, we can predict *Ascaris* inactivation based on exposure time and concentration.
Predict time and concentration requirements for complete inactivation

Uncharged $n$-Butyric Acid Concentration (mM) vs. Exposure Time (d)

Uncharged $n$-Caproic Acid Concentration (mM) vs. Exposure Time (d)
Predict time and concentration requirements for complete inactivation

<table>
<thead>
<tr>
<th>Acid</th>
<th>Exposure Time (d)</th>
<th>Predicted Uncharged Concentration Required to Reduce Viability Below Detection (0.2%) (mM)</th>
<th>95% Confidence Interval (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Butyric</td>
<td>2</td>
<td>529</td>
<td>506-553</td>
</tr>
<tr>
<td>n-Butyric</td>
<td>6</td>
<td>354</td>
<td>234-475</td>
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<tr>
<td>n-Butyric</td>
<td>12</td>
<td>276</td>
<td>236-316</td>
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<tr>
<td>n-Butyric</td>
<td>20</td>
<td>163</td>
<td>158-169</td>
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<tr>
<td>n-Caproic</td>
<td>2</td>
<td>32</td>
<td>31-33</td>
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<td>n-Caproic</td>
<td>6</td>
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<td>19-22</td>
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<tr>
<td>n-Caproic</td>
<td>12</td>
<td>13</td>
<td>11-16</td>
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<tr>
<td>n-Caproic</td>
<td>20</td>
<td>13</td>
<td>12-15</td>
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</tbody>
</table>
# Model parameters

<table>
<thead>
<tr>
<th>Acid</th>
<th>Exposure Time (d)</th>
<th>a (std error)</th>
<th>p-value a</th>
<th>b (std error)</th>
<th>p-value b</th>
<th>Mean Square Error</th>
<th>Mean Absolute Error</th>
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</thead>
<tbody>
<tr>
<td>Butyric</td>
<td>2</td>
<td>-0.0519 (0.00480)</td>
<td>4.71*10^(-6)</td>
<td>409 (2.23)</td>
<td>8.75*10^(-16)</td>
<td>0.000981</td>
<td>0.0282</td>
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<tr>
<td>Butyric</td>
<td>6</td>
<td>-0.0448 (0.0203)</td>
<td>0.0582</td>
<td>216 (14.1)</td>
<td>3.23*10^(-7)</td>
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<td>0.0641</td>
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<tr>
<td>Butyric</td>
<td>12</td>
<td>-0.0462 (0.00707)</td>
<td>0.000182</td>
<td>141 (3.85)</td>
<td>3.36*10^(-10)</td>
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<td>0.0303</td>
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<tr>
<td>Butyric</td>
<td>20</td>
<td>-0.0101 (0.00418)</td>
<td>9.26*10^(-9)</td>
<td>102 (0.388)</td>
<td>2*10^(-16)</td>
<td>2.58*10^(-5)</td>
<td>0.00315</td>
</tr>
<tr>
<td>Caproic</td>
<td>2</td>
<td>-0.954 (0.0927)</td>
<td>6.87*10^(-6)</td>
<td>25.1 (0.200)</td>
<td>1.83*10^(-14)</td>
<td>0.000987</td>
<td>0.0171</td>
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<tr>
<td>Caproic</td>
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<td>-1.09 (0.199)</td>
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<td>15.2 (0.377)</td>
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<td>Caproic</td>
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<td>-2.24 (1.11)</td>
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<td>10.6 (0.179)</td>
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<td>Caproic</td>
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<td>-1.17 (0.188)</td>
<td>0.000254</td>
<td>8.08 (0.103)</td>
<td>7.61*10^(-13)</td>
<td>0.00146</td>
<td>0.024</td>
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</table>
Reverse Beta-Oxidation

Step 1: ethanol

6 ethanol $\rightarrow$ 6 acetaldehyde $\rightarrow$ 1 acetate
6 NAD$^+$ $\rightarrow$ 6 NADH $\rightarrow$ ATP
6 acetyl-CoA $\rightarrow$ acetyl-Pi

Step 2: acetate

5 acetate $\rightarrow$ 5 acetyl-CoA $\rightarrow$ 5 CoA
5 acetoacetyl-CoA $\rightarrow$ 5 NAD(P)H $\rightarrow$ 5 NAD(P)
5 3-hydroxybutyryl-CoA $\rightarrow$ 5 H$_2$O
5 crotionyl-CoA $\rightarrow$ 5 NAD$^+$

Step 2: n-butyrate

Reactions in Step 1: ethanol lead to 5 mol acetyl-CoA entering pathway

5 n-caproate $\rightarrow$ 5 3-keto-hexanoyl-CoA $\rightarrow$ 5 NAD(P)H $\rightarrow$ 5 NAD(P)
5 n-butyrate $\rightarrow$ 5 hexanoyl-CoA $\rightarrow$ 5 3-hydroxyhexanoyl-CoA $\rightarrow$ 5 H$_2$O
3 hex-2-enoyl-CoA $\rightarrow$ 2 H$_2$

Slide by: C. Spirito
Reverse Beta-Oxidation: C2 to C4

\[
\text{6 ethanol} \rightarrow \text{6 acetaldehyde} \rightarrow \text{6 acetyl-CoA} \rightarrow \text{acetyl-Pi}
\]

\[
\text{6 acetyl-CoA} \rightarrow \text{5 acetyl-CoA} \rightarrow \text{5 acetyl-CoA} \rightarrow \text{5 acetyl-CoA} \rightarrow \text{5 acetyl-CoA}
\]

\[
\text{5 acetyl-CoA} \rightarrow \text{5 acetoacetyl-CoA} \rightarrow \text{5 3-hydroxybutyryl-CoA} \rightarrow \text{5 crotonyl-CoA}
\]

\[
6 \text{ ethanol} + 4 \text{ acetate}^- \rightarrow 5 \text{ n-butyrate}^- + \text{H}^+ + 2\text{H}_2 + 4\text{H}_2\text{O}
\]

Slide by: C. Spirito
## Concentrations for uncharged acid data

<table>
<thead>
<tr>
<th>Treatment #</th>
<th>pH</th>
<th>Uncharged Butyric Acid (mM)</th>
<th>Uncharged Caproic Acid (mM)</th>
<th>Total Butyrate (mM)</th>
<th>Total Caproate (mM)</th>
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</thead>
<tbody>
<tr>
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<td>100</td>
<td>10</td>
<td>100</td>
<td>10.0</td>
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<tr>
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<td>135</td>
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<td>17</td>
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<td>4</td>
<td>135</td>
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<td>4</td>
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<td>9</td>
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<td>236</td>
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<td>251</td>
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<td>5</td>
<td>240</td>
<td>24</td>
<td>603</td>
<td>55.6</td>
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</tbody>
</table>
Fermentation data

- Acetate
- Propionate
- i-Butyrate
- n-Butyrate
- i-Valerate
- n-Valerate
- n-Caproate

Carboxylate Concentration (mM) vs. Time (d)
Ascaris eggs exposed to *n*-butyric acid and *n*-caproic acid in fecal sludge

19 Day Exposure

- Treatment 1: Spiked acids and low pH
- Treatment 2: No acids and low pH
- Treatment 3: Spiked acids and unadjusted pH
- Treatment 4: No acids and unadjusted pH
- Treatment 5: Autoclaved fecal waste, no acids, unadjusted pH