New Trends in Silage Microbiology

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Outlines

• Mission unaccomplished
  Why further study is necessary?

• Application of culture-independent analyses
  QPCR
  DGGE

• Toward a new era
  Next generation sequencing
Mission unaccomplished
Why further research is necessary?

New breed Area expansion
Device improvement Various DM level
Aerobic instability
Nutrition & Health

We should encourage desirable LAB growth in the anaerobic phase, whereas need to suppress any microbial activities after silo opening. Plus probiosis ...
Anaerobic and aerobic metabolism by silage microorganisms

Anaerobic

Lactate, acetate, ethanol, mannitol, CO2

Ethanol, CO2

Acetic acid bacteria

Lactic acid bacteria

Clostridia

Enterobacteria

Propionic acid bacteria

Yeast

Molds

Propionate, acetate, CO2

Butyrate, H2, CO2

Lactate, acetate, ethanol, 2,3-butanediol, CO2, endotoxins

CO2, H2O

Bacilli

Water soluble carbohydrates

Lactate, Acetate, Ethanol

Aerobic

CO2, H2O

CO2, H2O, Mycotoxins

(Merry and Davies, 1999)
Application of culture-independent analyses
<table>
<thead>
<tr>
<th>Tool</th>
<th>Size (bp)</th>
<th>Sensitivity</th>
<th>Advantages/Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>QPCR</td>
<td>100–150</td>
<td>$10^1$–$10^3$ cfu/g</td>
<td>Sensitive, quantitative Targeted technique</td>
</tr>
<tr>
<td>DGGE</td>
<td>100-300</td>
<td>$10^2$–$10^3$ cfu/g</td>
<td>Single-nucleotide fragment separation Qualitative, technically difficult</td>
</tr>
<tr>
<td>TRFLP</td>
<td>1,000+</td>
<td>$10^2$ cfu/g</td>
<td>High-throughput, pseudoquantitative</td>
</tr>
<tr>
<td>Illumina</td>
<td></td>
<td></td>
<td>Lower taxonomic resolution</td>
</tr>
<tr>
<td>HiSeq</td>
<td>50-150</td>
<td>$10^9$ reads/run</td>
<td>Superior sequence coverage, lowest sequencing cost</td>
</tr>
<tr>
<td>MiSeq</td>
<td>50-300</td>
<td>$10^7$ reads/run</td>
<td>Shorter fragment length, theoretically lower taxonomic resolution</td>
</tr>
</tbody>
</table>
DGGE – a community profiling tool –

- Band excision & DNA extraction
- PCR without GC clamp
- DNA purification & cloning
- Colony PCR
- Plasmid DNA purification
- DNA sequence
- Homology search by BLAST
Molasses addition can encourage aerobic stability?

Prompt spoilage without fermentation

Good stability regardless of fermentation patterns

60-day silage

Untreated

+ Molasses
Association of *Lactobacillus fructivorans* was suggested

1. *Pantoea* sp.
2. *Oceanobacillus picturae*
3. *Lactobacillus fructivorans*
4. *Enterococcus mundtii*
5. *Lactobacillus fructivorans*
6. *Lactobacillus fructivorans*
7. *Lactobacillus fructivorans*
8. *Pantoea* sp.
9. *Methylobacterium populi*
10. *Pantoea vaqans*
11. *Pantoea* sp.
12. *Enterococcus sulfureus*
LF has never been isolated because of MRS medium

<table>
<thead>
<tr>
<th>MRS medium</th>
<th>g/L</th>
<th>LIS medium</th>
<th>g/L</th>
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<tbody>
<tr>
<td>Peptone</td>
<td>10.0</td>
<td>Liver (fresh)</td>
<td>400 g</td>
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<tr>
<td>Lab-Lemco powder</td>
<td>8.0</td>
<td>Sake</td>
<td>400mL</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4.0</td>
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<tr>
<td>Glucose</td>
<td>20.0</td>
<td></td>
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</tr>
<tr>
<td>Sorbitan monoleate</td>
<td>1mL</td>
<td></td>
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</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>2.0</td>
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</tr>
<tr>
<td>Sodium citrate 3H2O</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triammonium citrate</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium sulphate 7H2O</td>
<td>0.2</td>
<td></td>
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</tr>
<tr>
<td>Manganese sulphate 4H2O</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Did not work

Successful!

- *Lactobacillus acidipiscis* was predominant in MRS medium isolates.
- >70% was *Lactobacillus fructivorans* in LIS medium isolates.
DGGE— a community profiling tool —

Electrophoresis

Denaturing gradient

Low

High

Band excision & DNA extraction

PCR without GC clamp

DNA purification & cloning

Colony PCR

Plasmid DNA purification

DNA sequence

Homology search by BLAST

DGGE = community profiling tool
Evaluation of LAB survival in the cow gut

**Probiotics**

**Silage inoculants**
- *Lactobacillus plantarum*
- *Lactobacillus acidophilus*
- *Lactobacillus casei*
- *Lactobacillus buchneri*
- *Enterococcus faecium*

**Rumen**
- *Lactobacillus vitulinus*
- *Lactobacillus ruminis*
- *Lactobacillus johnsonii*
- *Lactobacillus murinus*
- *Streptococcus bovis*

<table>
<thead>
<tr>
<th></th>
<th>%</th>
<th></th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td>Rumen</td>
<td></td>
<td>Feces</td>
<td></td>
</tr>
<tr>
<td><em>Prevotella</em></td>
<td>18.2</td>
<td><em>Clostridium</em></td>
<td>19.7</td>
</tr>
<tr>
<td><em>Succinivibrio</em></td>
<td>11.8</td>
<td><em>Bacteroides</em></td>
<td>10.5</td>
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<tr>
<td><em>Bacteroidales</em></td>
<td>11.7</td>
<td><em>Bacteroidales</em></td>
<td>4.83</td>
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<tr>
<td><em>Butyrivibrio</em></td>
<td>4.34</td>
<td><em>Prevotella</em></td>
<td>4.38</td>
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<tr>
<td><em>Bacteroides</em></td>
<td>3.78</td>
<td><em>Ruminococcus</em></td>
<td>3.73</td>
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<tr>
<td><em>Clostridium</em></td>
<td>3.14</td>
<td><em>Eubacterium</em></td>
<td>3.25</td>
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<tr>
<td><em>Eubacterium</em></td>
<td>2.08</td>
<td><em>Alistipes</em></td>
<td>3.12</td>
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<tr>
<td><em>Ruminococcus</em></td>
<td>0.85</td>
<td><em>Rhizobium</em></td>
<td>1.06</td>
</tr>
</tbody>
</table>

Callaway et al. (2010)
PCA indicated difficulty of survival
Group-specific analysis to detect LAB species in the gut

22 Lactobacillus acetotolerans
23 Lactobacillus pontis
24 Lactobacillus vini
25 Lactobacillus reuteri
26 Uncultured bacterium
27 Lactobacillus salivarius
28 Uncultured bacterium
29 Uncultured bacterium
30 Uncultured bacterium
31 Lactobacillus acetotolerans
32 Uncultured bacterium
33 Lactobacillus acetotolerans
34 Lactobacillus fermentum
35 Lactobacillus acetotolerans
36 Lactobacillus pontis
37 Lactobacillus fermentum
38 Lactobacillus pontis
39 Uncultured bacterium
40 Lactobacillus vini
41 Lactobacillus amylolyticus
42 Lactobacillus crispatus
43 Lactobacillus crispatus
44 Uncultured bacterium
Quantitative PCR – a targeted tool –

Total population quantification
- Bacteria 16S rRNA gene
- Fungi 26S rRNA gene

Genus specific quantification
- *Lactobacillus*
- *Clostridium* ......

Species specific quantification
- *Lactobacillus plantarum*
- *Lactobacillus casei*
- *Lactobacillus buchneri*
- *Lactococcus lactis* ......

Functional gene quantification
- Histidine decarboxylase (*hdc*)
- Glycerol dehydratase (*gd*) ......

Fluorescence

Cycle

Ct

Log quantity
Combination of qualitative DGGE and QPCR

1. Uncultured bacteria
2. *Lactobacillus brevis*
3. Uncultured bacteria
4. *Pediococcus pentosaceus*
5. *Lactococcus lactis*
6. *Lactobacillus buchneri*
7. Uncultured bacterium
8. Uncultured bacterium
9. *Lactobacillus plantarum*
10. Uncultured bacterium
11. Uncultured bacterium
12. Uncultured bacterium
13. Uncultured bacterium
14. *Bacillus* sp.
15. *Bacillus* sp.
16. Uncultured bacterium

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Lactate (g/kgDM)</th>
<th>Acetate (g/kgDM)</th>
<th>QPCR (log cfu/g)</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Day 0</td>
<td>4.12</td>
<td>3.88</td>
<td>1.61</td>
<td>5.51</td>
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<tr>
<td>+ 3</td>
<td>4.46</td>
<td>3.24</td>
<td>0.77</td>
<td>5.87</td>
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<tr>
<td>+ 7</td>
<td>6.05</td>
<td>1.14</td>
<td>0.19</td>
<td>5.23</td>
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<tr>
<td><em>L. buchneri</em>-inoculated</td>
<td></td>
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<td></td>
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<tr>
<td>Day 0</td>
<td>4.06</td>
<td>3.68</td>
<td>1.99</td>
<td>8.10</td>
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<tr>
<td>+ 3</td>
<td>4.11</td>
<td>3.65</td>
<td>1.30</td>
<td>8.57</td>
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<tr>
<td>+ 7</td>
<td>4.72</td>
<td>1.99</td>
<td>0.63</td>
<td>8.93</td>
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</table>
Toward a new era
– next generation sequencing –
Thousands of species are detectable by NGS

Sequences per sample

<table>
<thead>
<tr>
<th>Pre-ensiled grass</th>
<th>Pre-ensiled corn</th>
<th>Pre-ensiled alfalfa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa silage 3</td>
<td>Alfalfa silage 1</td>
<td>Alfalfa silage 2</td>
</tr>
<tr>
<td>Grass silage 1</td>
<td>Grass silage 3</td>
<td>Grass silage 2</td>
</tr>
<tr>
<td>Corn silage 1</td>
<td>Corn silage 3</td>
<td>Corn silage 2</td>
</tr>
</tbody>
</table>

Sequences by synthesis

Thousands of species are detectable by NGS.

- Pre-ensiled grass
- Pre-ensiled corn
- Pre-ensiled alfalfa
- Alfalfa silage 3, 1, 2
- Grass silage 1, 3, 2
- Corn silage 1, 3, 2

Observed species

- Pre-ensiled grass
- Pre-ensiled corn
- Pre-ensiled alfalfa
- Alfalfa silage 3, 1, 2
- Grass silage 1, 3, 2
- Corn silage 1, 3, 2

Sequences by synthesis
Family level quantification by NGS

<table>
<thead>
<tr>
<th>Family</th>
<th>PE Silage</th>
<th>PE Silage</th>
<th>PE Silage</th>
<th>PE Silage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilted grass</td>
<td></td>
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</tr>
<tr>
<td>Whole crop corn</td>
<td></td>
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<tr>
<td>Wilted alfalfa</td>
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<tr>
<td>Acetobacteraceae</td>
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<tr>
<td>Actinomycetales</td>
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<td>Alcaligenaceae</td>
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<td>Aurantimonadaceae</td>
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<td>Bacillaceae</td>
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<td>Beutenbergiaceae</td>
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<td>Comamonadaceae</td>
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<td>Coxillaceae</td>
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<tr>
<td>Cytophagaceae</td>
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<tr>
<td>Enterobacteriaceae</td>
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<td>Enterococcaceae</td>
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<td>Flavobacteriaceae</td>
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<td>Leuconostocaceae</td>
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<td>Methylobacteriaceae</td>
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<td>Nocardiaceae</td>
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<tr>
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<td>Phyllobacteriaceae</td>
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<td>Planococcaceae</td>
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<tr>
<td>Streptophyta</td>
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<tr>
<td>Sphingomonadaceae</td>
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</tbody>
</table>
Qualitative DGGE for the same silages

1. Uncultured sp.
2. Weissella sp.
3. Weissella sp.
4. Weissella sp.
5. Staphylococcus sp.
6. Enterobacter sp.
7. Leuconostoc sp.
8. Weissella sp.
9. Leuconostoc sp.
10. Pediococcus sp.
11. Pseudomonas sp.
12. Uncultured sp.
13. Uncultured sp.
14. Methylobacterium sp.
15. Pseudomonas sp.
16. Uncultured sp.
17. Uncultured sp.
18. Pediococcus sp.
19. Uncultured sp.
20. Uncultured sp.
21. Agrobacterium sp.
22. Klebsiella sp.
23. Streptomyces sp.
24. Staphylococcus sp.
25. Enterococcus sp.
26. Enterococcus sp.
27. Enterococcus sp.
28. Uncultured sp.
29. Enterococcus sp.
30. Terribacillus sp.
31. Paracoccus sp.
32. Agrobacterium sp.
33. Uncultured sp.
34. Sanguibacter sp.
35. Acetobacter sp.
36. Klebsiella sp.
37. Lactobacillus sp.
38. Lactobacillus sp.

PE Silage
Wilted grass
Whole crop corn
Wilted alfalfa
DGGE properly detects major bacteria detected by NGS

**Bacteria Detected by DGGE and NGS**

- **Leuconostocaceae**
- **Lactobacillaceae**
- **Enterococcaceae**
- **Pseudomonadaceae**
- **Enterobacteriaceae**
- **Xanthomonadaceae**
- **Lactobacillaceae**
- **Enterobacteriaceae**

**Sample Types**

- PE Silage
- Wilted grass
- Whole crop corn
- Wilted alfalfa
Proteins conserved in all species

Proteins unique to L. vaccinostercus group

Proteins unique to L. hokkaidonensis

**Lactobacillus hokkaidonensis**
- A cold-tolerant new LAB species -

Whole genome analysis will reveal unique metabolism
Silage with unknown microbial communities

Whole community profiling
NGS/DGGE

Diversity statistics
PCA / UPGMA

Strain profiling
Species level identification
Whole genome analysis

QPCR
Total population
Genus specific
Species specific
Functional gene

Biochemical/chemical profiling

Conclusion – Integrated approach is the best strategy –

Bacillus subtilis
Enterococcus sulfureus
Enterococcus lactis
Lactobacillus rhamnosus
Lactobacillus hilgardii
Lactobacillus brevis
Lactobacillus plantarum
Lactobacillus kefiri
Lactobacillus buchneri
Lactobacillus paraffermigeris
Lactobacillus dieterleii
Lactobacillus fructivorans
This study
Weissella cibaria
Weissella paramesenteroides
Weissella paramesenteroides
Lactobacillus lactis
Pediococcus acidilactici
Pediococcus pentosaceus

Weissella paramesenteroides
Lactobacillus lactis

Fluorescence

Cycle