Detection of spore-forming bacteria in dairy products

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Harvesting and ensiling

Silage consumption

Silage $<10^2 - 10^7$

Grass $<10^2 - 10^4$

Cow

Soil $<10^2 - 10^5$

Faeces $<10^2 - 10^8$

Manure fertilization

Manure storage $<10^2 - 10^7$

Milk $<10^1 - 10^2$

Defecation

Udder contamination
Eliminating thermodurics to improve the quality of powdered dairy ingredients

- (A) develop methods to facilitate the rapid identification of these bacteria,
- (B) identify the industrial cleaning-in-place agents that work most effectively against these microbes and
- (C) reveal food-grade antimicrobials which can (i) control the renewed build-up of these bacteria during processing and (ii) prevent their outgrowth when used as ingredients.
Thermoduric bacteria

- *Bacillus cereus* spp. – Gram positive, spore forming, toxin producing food borne pathogen

- Sulfite reducing *Clostridia* spp. – Gram positive anaerobic spore forming food borne pathogens (SRCs)

Problem: Ubiquitous in nature – FOUND EVERYWHERE!
What are SRCs

- Group of phenotypically distinctive sporeformers belonging to the order *Clostridiales*

- Distinguished by their ability to reduce sulphite to sulphide under anaerobic conditions

- Multiple phenotype specific agar assays designed to detect SRCs

- All rely on the reduction of ferric sulphite to iron sulphide, and the accompanying colour change
Why are SRCs important

- *Clostridium* sp. are found widespread throughout the dairy farm environment
- Sporeformers can survive commercial pasteurisation
  
  Germination later

- Include pathogenic and spoilage associated species
  
  *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium tetani*, *Clostridium butyricum* & *Clostridium beijerincki*

- Used as indicator organisms in the food industry
SRCs: Use of molecular approaches to assess the factors which impact on the biota of milk

Focus on farm level and milk at-farm
SRC are poorly defined – initial task to isolate SRC from culture and sequence them to identify them!!
90% are clostridia, but some others not recognised as SRC – B. licheniformis

- To analyze the composition of the sulphate-reducing Clostridium (SRCs) and sulphate-reducing bacteria (SRBs) in milk
- Progress: Identify common gene clusters between isolates; design primers
- Use of culture-independent approaches to assess/evaluate the impact of a variety of factors (including seasonality and storage temperature) on the microbiota of milk
- Progress: Currently being done
Surveillance of powders

- Working with dairy processors in Ireland
- Have received some SRC-containing dairy powders and cheese (as well as colonies of agar plates)
- Tested them according to protocols provided
- Isolated pure cultures of SRCs, stocked and identified isolates by sequencing the 16S rRNA gene
Molecular assay for detection of SRCs

- qPCR based assay targeting genes responsible for this phenotype

- Isolate and identify more SRCs to help identify common genes

- Target these genes to make a more rapid and specific phenotypic assay

- Have identified a possible target gene cluster, but work is on-going

- Early PCR results
Eliminating thermodurics to improve the quality of powdered dairy ingredients

- Focused on detection of aerobic spores in powders/milk

- Objectives
  - 1) Survey the species of spore-forming bacteria present in powdered dairy ingredients generated by Irish Dairy Companies and generate a rapid real-time PCR assay to detect, differentiate between and quantify spore-forming bacteria
  - 2) Identification of food-grade antimicrobials with activity against spore-forming bacteria
  - 3) Studies on biofilm formation and control in laboratory scale reactors
  - 4) Develop approaches to prevent the outgrowth of spore-forming bacteria during secondary processing
Culture methods

- Culture methods compared –
- MYP (Mannitol Egg Yolk Polymyxin Agar) v Bacarra
Analysis

- Raw samples – not pasteurised
- Serially diluted
- Plated on MYP
- Incubated at 32°C for 48 hours
- Confirmation on blood agar – β haemolysis
- Isolated for 16S rRNA identification
Identify 10 milk sample isolates from MYP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Identified species (Homology)</th>
<th>BC species (Homology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Macrococcus sp. KW16 (99%)</td>
<td>Bacillus mycoides strain JP44SK50 (94%)</td>
</tr>
<tr>
<td>2</td>
<td>Staphylococcus sp. ChDC B592 (99%)</td>
<td>Bacillus thuringiensis serovar konukian str. 97-27 (93%)</td>
</tr>
<tr>
<td>3</td>
<td>Enterobacter aerogenes (95%)</td>
<td>Bacillus cereus strain M-7 (81%)</td>
</tr>
<tr>
<td>4</td>
<td>Pseudomonas gessardii strain AMHSOL259 (99%)</td>
<td>Bacillus weihenstephanensis strain CtST10.5 (88%)</td>
</tr>
<tr>
<td>5</td>
<td>Pseudomonas trivialis strain KOPRI 25674 (99%)</td>
<td>Bacillus weihenstephanensis strain CtST10.5 (88%)</td>
</tr>
<tr>
<td>6</td>
<td>Yersinia sp. UA-JF0918 (100%)</td>
<td>Bacillus weihenstephanensis strain CtST10.5 (85%)</td>
</tr>
<tr>
<td>7</td>
<td>Uncultured bacterium clone S11_049 (95%)</td>
<td>Bacillus anthracis strain TMPTTA CASMB 6 (87%)</td>
</tr>
<tr>
<td>8</td>
<td>Enterococcus faecalis strain L3B1K3 (99%)</td>
<td>Bacillus cereus strain AGP-03 (92%)</td>
</tr>
<tr>
<td>9</td>
<td>Staphylococcus simulans strain QTR-52 (98%)</td>
<td>Bacillus cereus strain LCB46 (93%)</td>
</tr>
<tr>
<td>10</td>
<td>Lactococcus lactis subsp. cremoris strain RU36-7 (99%)</td>
<td>Bacillus anthracis strain HDDMM10 (86%)</td>
</tr>
</tbody>
</table>

No isolate was identified as a *Bacillus cereus* species.
Compare MYP to BACARA agar

- Bacara agar – FDA recommended
- Pre-poured plates are bought from Biomerieux
- Incubated at 32°C for 24 hours
Compare MYP to BACARA agar
## Compare MYP to BACARA agar

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacara identified species</th>
<th>MYP identified species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Bacillus cereus</em> strain</td>
<td><em>Staphylococcus hominis</em> strain</td>
</tr>
<tr>
<td>2</td>
<td><em>Bacillus mycoides</em> strain</td>
<td><em>Lactococcus lactis</em> strain</td>
</tr>
<tr>
<td>3</td>
<td><em>Bacillus thuringiensis</em></td>
<td><em>Bacillus cereus</em> strain BE4-1 <em>Bacillus cereus</em> strain HKS1-1</td>
</tr>
<tr>
<td>4</td>
<td><em>Bacillus cereus</em> strain</td>
<td><em>Bacterium M133-5</em></td>
</tr>
<tr>
<td>5</td>
<td>Not enough DNA</td>
<td><em>Bacillus mycoides</em> strain</td>
</tr>
<tr>
<td>6</td>
<td><em>Enterococcus</em></td>
<td><em>Lactococcus</em></td>
</tr>
</tbody>
</table>
Conclusion – this section

- Bacara agar is more selective for *Bacillus cereus* group, especially for raw milk samples
Non-culture methods

Several existing PCR methods based on toxin detection.

- Duopath® Cereus enterotoxins - confirms the presence of the diarrhoeal enterotoxins of *B. cereus*. The kit has a detection limit of 100 CFU/g

- *B. cereus* detection kit - target the *B. cereus*-specific gene GroEL.

- *B. cereus* real-time PCR kit - real-time PCR kit detects the presence of an amplified *B. cereus* DNA fragment and toxins are identified

Develop a multiplex assay combining these method
Will detect *B. cereus* but not other *B. cereus* group bacteria.
Thank you for your attention