Persister cells – cells that keep on giving

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Resistance and Persistance

• Resistance = populations of cells that survive antimicrobial treatment

• Persistence = a proportion of cells that survive antimicrobial treatment
Mechanism of bacterial persistence

Persistent cells
- Stable tolerant survivors (genotypic heterogeneity)
- Temporary tolerant survivors (phenotypic switching)

What is persistence?

- Persistence = long term occurrence of genetically indistinguishable strains in the same environment

- Months or Years?

- PFGE, MLST, WGS ??

- Conveyor belt? Same room? Same factory?

- Very broad description
Two current models of persistence

Persistence

Random process

Genotypic and phenotypic features

?
First Study
Persistent *L. monocytogenes* from a manufacturing plant

- Isolates taken from the manufacturing plant environment

- “Persistent” types based on frequent analysis of molecular fingerprinting types
Our Approach – focus on *Listeria monocytogenes*

- Genetic approach
  - IFR Norwich, UK
  - 48 strains

- Phenotypic approach
  - Wageningen University, The Netherlands
  - 8 persistent strains + 7 sporadic + 1 outbreak strain
Genotype Approach - WGS

• 10 persistent strains
  ➢ Isolated from food environments
  ➢ 4 persistent pulsotypes

• 32 sporadic strains
  ➢ Isolated from food processing environment

• 5 other
  ➢ Human isolates, outbreak isolates, mutant strain

Illumina, MiSeq, 250bp read length
Genotype Approach – From DNA to Data

DNA → Sequencing → De novo Assembly (SPAdes) → Visualization (Artemis and MAUVE) → Annotation (PROKKA) → Comparison:

- core / accessory genome (ROARY)
- SNP (Parsnp)
- FFP
Some differences associated with mobile genetic elements

Differences might be multifactorial or based on subtle differences in the core genome
Phenotype approach – 16 strains

**Persistent isolates**

| Pulsotype 3814 | 15A04 (plant II) |
|               | 27A05 (plant I) |
| Pulsotype 5132 | 15G01 (mutant parent, plant I) |
|               | 16J10 (plant I) |
| Pulsotype 5588 | 32C06 (plant III) |
|               | 33H04 (plant III) |
| Pulsotype 6502 | 15A07 (plant II) |
|               | 31H06 (plant II) |

**Sporadic + outbreak isolate**

| 15B09 (plant I) |
| 15D07 (plant I) |
| 16J08 (plant I) |
| 19B07 (plant I) |
| 15G10 (plant II) |
| 17A02 (plant II) |
| 16H02 (plant IV) |
| 16A01 food outbreak |

**Environmental isolates**
Phenotype Approach - Tests

- Biofilm formation
  - CV-staining
  - Plating
- Heat treatment
  - Plating
  - Flow cytometry
- Motility
- Growth
- Survival on dry surface
  - Planktonic cells
  - Biofilm cells
Phenotype Approach – Biofilm formation

Conditions tested:
20°C (24, 48 h)
30°C (24, 48 h)
Medium: BHI

Crystal violet staining
- No indication about viable cells
- Stains any organic matter

Cell enumeration by plating
- Detects viable cells
- Gives an indication about living cells in the biofilm
Phenotype Approach – Biofilm formation at 20°C

Minimal biofilm formation
Cell counts between 3-6 log CFU/well
No specific persistent behaviour
Phenotype Approach – Biofilm Formation at 30°C

5 persistent strains and 1 sporadic strain show higher cell count and biofilm mass after 24 hours

6 persistent strains and 1 sporadic strain show higher cell count and biofilm mass after 48 hours
Phenotype Approach – Heat resistance

- Heat treatment at 58°C for 5min
  - 5 min recovery
  - 2 h recovery
- Aim: To identify ability of heat treated strains to recover
- Plating and Flow cytometry
Phenotype Approach
- Principle of Flow Cytometry

Flow Cytometry

Sheath fluid → Sample (stained cells in suspension) → Nozzle

Hydrodynamic Focusing
Cells pass through in "single file"

Laser light source → Fluorescence emitted from stained cells detected → Forward and side scattered light from all cells detected
Phenotype Approach
- Flow Cytometry Output

Experiment Name: Bcereus standard PI
Specimen Name: Coop Jessika_20150624
Tube Name: Listeria 15G01_0n_PBS_dead_PI
Record Date: Jun 25, 2015 11:12:09 AM
$OP: Administrator
GUID: 1f4aa17d-e608-4b42-8945-e318f4ba6b5b

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<thead>
<tr>
<th>Population</th>
<th>#Events</th>
<th>%Parent</th>
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<tbody>
<tr>
<td>Total cells</td>
<td>50,000</td>
<td>100.0</td>
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<tr>
<td>Dead listeria</td>
<td>49,650</td>
<td>99.3</td>
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<tr>
<td>&quot;Damaged&quot;</td>
<td>292</td>
<td>0.6</td>
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<tr>
<td>Alive</td>
<td>52</td>
<td>0.1</td>
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Phenotype Approach

Results

Plating

Surviving cells [log %]

Living cells [log %]

Total (persistent/sporadic)

3 (2/1)

7 (4/3)

6 (2/4)

Flow cytometer

6 (4/2)

7 (4/3)

3 (0/3)
Results

- Majority of the strains had significantly lower CFU/ml after heat treatment (ANOVA, $p \leq 0.001$), but no significant difference between the mean values of the difference at 5min and 2h (ANOVA, $p=0.232$)

- 2 factor ANOVA with replication

<table>
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<th>persistent</th>
<th>sporadic</th>
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<tr>
<td>Average t0</td>
<td>9.09465053</td>
<td>8.92693031</td>
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<tr>
<td>variance</td>
<td>0.01680262</td>
<td>0.00252948</td>
</tr>
<tr>
<td>Average t5min</td>
<td>8.10540189</td>
<td>7.65350223</td>
</tr>
<tr>
<td>variance</td>
<td>0.00071655</td>
<td>0.01332252</td>
</tr>
<tr>
<td>Average t2h</td>
<td>8.01439826</td>
<td>7.61914735</td>
</tr>
<tr>
<td>variance</td>
<td>0.00090569</td>
<td>0.00023951</td>
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</table>

Persistent/sporadic $p \leq 0.001$
Interaction $p=0.081$
Survival on dry surfaces

**Biofilm cells**

Overnight culture
TSBYE
37°C

**Planktonic cells**

24 hours
BHI
25°C

48 hours
BHI
30°C

Incubation at 25°C

Survival on Day 0, 1, 2, 5, 7 and 14
Results

- Cell numbers decreased sharply on Day 1 compared to initial concentration
  - Planktonic cells reduction of 1.93 log CFU/well
  - Biofilm cells reduction of 1.83 log CFU/well
- Survival after 14d
  - Planktonic cells reduction ranging between 3.01 – 5.29 log CFU/well
  - Biofilm cells reduction ranging between 2.57 – 5.05 log CFU/well
  - Sporadic planktonic cultures highest reduction of 3.76 – 5.29 log CFU/well
  - Persistent biofilm cells lowest reduction ranging from 2.57 – 4.12 log CFU/well
Conclusions

• Unbalanced two factor ANOVA (Isolation and persistence/non-persistence)
  • Persistent strains form more biofilm than sporadic strains at 30°C after 48h incubation (CV 0.2 vs 0.12, p=0.039; cell numbers 6.62 log cfu/ml vs 6.30, p=0.028)
  • Initial percentage of cells alive (flow cytometry average 97% vs 96%, p=0.06)
  • Survival at Day 2 for biofilm cells (4.06 log cfu/well vs 3.27, p=0.074)
• No growth defects for all strains
• No genetic traits identified
• Representatives of each pulsotype behave similar
Principal component analysis (PCA)
Second study
Persister cells following Nisin treatment

• Selecting cells that survive increasing levels of nisin treatment
Gaps and limits in current studies of persister formation on food safety relevance:

• Inadequate number persisters during sampling in food environments
  Viable but Non-culturable cells – hard to detect

• Surface adhering ability (biofilm forming ability)  Can not explain the persistence

*Sanitizers: quaternary ammonium compounds, chlorine dioxide, peracetic acid

• The persistence following treatment with natural antimicrobials like bacteriocins has not been determined for *L. monocytogenes*;

<table>
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<tr>
<th>Product</th>
<th>Benefit</th>
<th>Application</th>
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<tbody>
<tr>
<td>Nisaplin</td>
<td>Anti-gram-positive</td>
<td>All types of foods: dairy, culinary, meat, bakery products and beverages</td>
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</table>
Research interests:

- What are the conditions that favour the *L. monocytogenes* persister formation in **planktonic form**?
- What are the conditions that favour the *L. monocytogenes* persister formation in a **biofilm matrix**?

- What **mechanisms are involved** in *L. monocytogenes* persisting?

**under high concentrations of nisin**

**Precondition:** Be able to collect adequate persister cells under nisin treatment

**Firsthand Task:**
Whether persister cells can be isolated following nisin treatment?
Biofilm screening of *L. monocytogenes* from foods and food related environments (48 isolates)

A1-20: AsureQuality Limited, NZ;
R1-R9: Plant & Food Research, NZ;
M1-M7: Albany campus of Massey university;
H1-12: Hills Lab (an independent NZ analytical testing centre).

**microtiter plate assay**

**The biofilm formation index (BFI):**

\[ \text{BFI} = \frac{(\text{AB} - \text{CW})}{G} \]

*AB*: attached bacteria biofilm
*CW*: blank wells
*G*: optical density of cells growth in suspended culture.

(strain M5 is the NCTC 7973 strain isolated from Guinea pig mesenteric lymph node)

- **strong** (≥1.10),
- **moderate** (0.70–1.09),
- **weak** (0.35–0.69)
Identify the presence of *L. monocytogenes* persister cells by dose-dependent killing of planktonic cells.

100µl blank/nisin  
+ 900µl overnight culture

![Graph](image)

Figure 3a. Concentration-dependent killing of *L. monocytogenes* A1 planktonic cells treated with nisin at concentrations of 0-75µg/ml at 30°C for 24 h.

- Tolerant to prolonged treatment with high dosed of bactericidal nisin;
- Genetically identical to susceptible bacteria;

![Graph](image)

Figure 3b. Six persister isolates from the A1 strain (AP1-AP6) which survived 24hrs treatment with 75µg/ml nisin in TSB were re-exposed to 75µg/ml nisin at 30°C for 24hrs.
What if we **resuspended** overnight culture cells in to new medium? 
and
How would the resuspended cells respond to nisin treatment?

Resuspend in TSB/

Diluted TSB

+Nisin treatments

Incubation 24hrs
The effect of nutrients on the production of *L. monocytogenes* persister cells

The re-suspension cells showed increased persistence;

Some components within the TSB medium could be a key mediator for *L. monocytogenes* persister formation

Figure 4a Dose-dependent killing of re-suspended cells of the A1 strain. The blue bars represent an

Nutrient limitation?
Whether limited nutrient condition favours persister formation?

1. Resuspend in TSB/Diluted TSB
2. Incubation first 24hrs
3. +Nisin treatments
4. Incubation second 24hrs
5. time 0
6. time 24
4 The effect of nutrients on the production of *L. monocytogenes* persister cells

**Delayed Nisin treatment**

- **Resuspend in TSB/ Diluted TSB**
- **Incubation first 24hrs**
- **Incubation second 24hrs**
- **time 0**
- **time 24**

**Immediate Nisin treatment**

- **Resuspend in TSB/ Diluted TSB**
- **+Nisin treatments**
- **Incubation 24hrs**

**Dormant state**
- Slow or no growth of the cells
- A slow metabolism

Figure 4a: Dose-dependent killing of re-suspended cells of the A1 strain. The blue bars represent an
What about persisters in biofilm following with nisin treatment?
Optimizing methods for obtaining *L. monocytogenes*persisters in a biofilm model

Matrix polymers

blank/ nisin treatment + formed biofilm on stainless steel coupon

Biofilm showed increased persistence

Genetically identical to susceptible bacteria;

Possiblity of increased persistence linking with the extracellular polymers structure of biofilm?
Hypotheses

- *L. monocytogenes* persister formation is dependent on the cell metabolic rate in planktonic form (nutrient factors, cellular factors)

- *L. monocytogenes* persister formation is influenced by specific features in a biofilm community (e.g. structure of the extracellular polymers)

- *L. monocytogenes* persister formation is due to the expression of specific genes in both the planktonic and biofilm communities.

Related mechanisms involved in persister formation

- Dormancy;
- Cell – cell communication (Quorum sensing);
- Toxin/antitoxin system;
- Efflux pump

Clinical relevance studies
Gene expression in persister cells

• Increased or decreased expression of genes is seen in presister cells

• This helps our understanding of how bacteria cope when exposed to stress (preservatives or sanitisers)

• How can we use this to avoid persister populations?
Select gene expression changes

• Stress response

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
<th>Increase/decrease</th>
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<tbody>
<tr>
<td>lmo1580</td>
<td>Universal stress protein</td>
<td>+ 2.89</td>
</tr>
<tr>
<td>lmo2004</td>
<td>Transcription regulator</td>
<td>- 4.91</td>
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</table>
Select gene expression changes

• Cell wall synthesis

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<tr>
<td>lmo0129</td>
<td>amidase</td>
<td>+4.09</td>
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<tr>
<td>lmo2714</td>
<td>Peptidoglycan bound protein</td>
<td>-3.44</td>
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Select gene expression changes

- DNA repair and damage

<table>
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<tr>
<td>lmo1975</td>
<td>DNA polymerase IV</td>
<td>-4.03</td>
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- No genes upregulated
Select gene expression changes

- ATP binding /transport system

<table>
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<th>Function</th>
<th>Increase/decrease</th>
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<td>lmo1636</td>
<td>ATP-binding protein</td>
<td>+3.58</td>
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<tr>
<td>lmo1730</td>
<td>Sugar transport</td>
<td>-3.49</td>
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Select gene expression changes

• Bacteria change their gene expression to cope with preservatives/sanitisers

• Suggests going into “lock down” or “sleep” until conditions improve

• A natural temporary protective mechanism

• Does this “evolve” into resistance?
Antimicrobial treatments

Persistence

Resistance

Food contamination

Understanding the mechanism of persister formation
What does this mean for us in the dairy industry

• Vary sanitisers used
• **Use heat treatment where possible**
• Ensure optimum strength of sanitisers/preservatives
• **Use multiple antimicrobial treatments**
Thank you!

Never underestimate the power of persistence.
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