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Dairy Australia Webinar

Persister cells – cells that keep on giving

Steve Flint

October 2017

THE ENGINE OF THE NEW NEW ZEALAND

Resistance and Persistance

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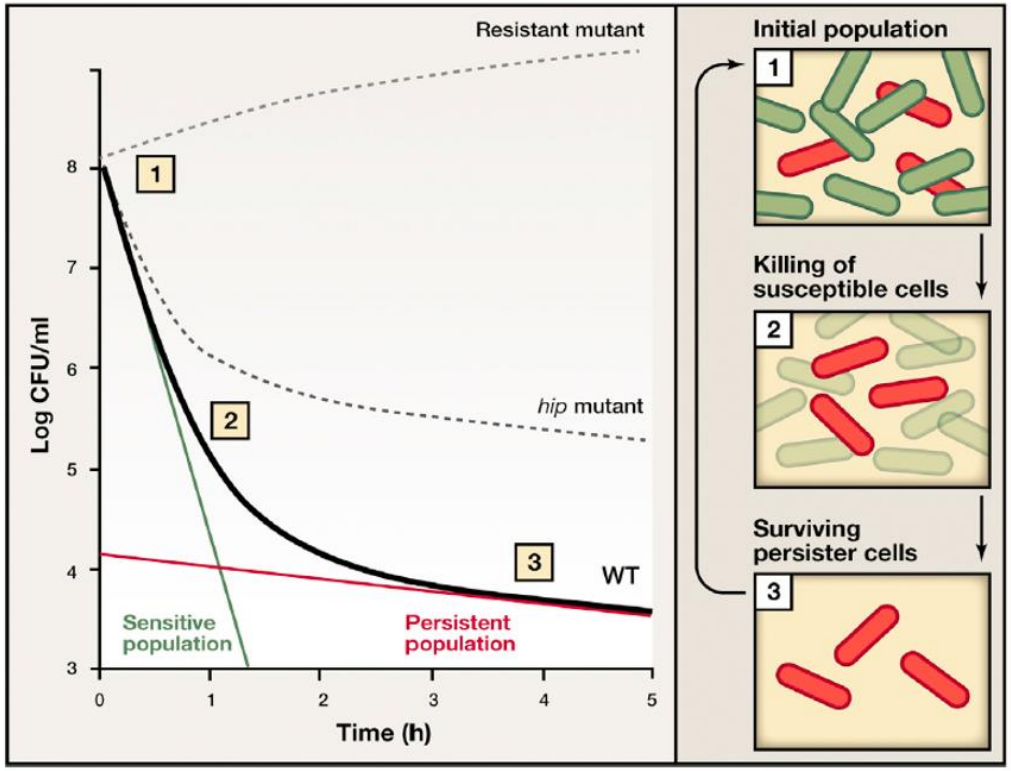
- Resistance = populations of cells that survive antimicrobial treatment

- 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)

Maisonneuve, E. and K. Gerdes, *Molecular mechanisms underlying bacterial persisters.* Cell, 2014. **157**(3): p. 539-48.

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

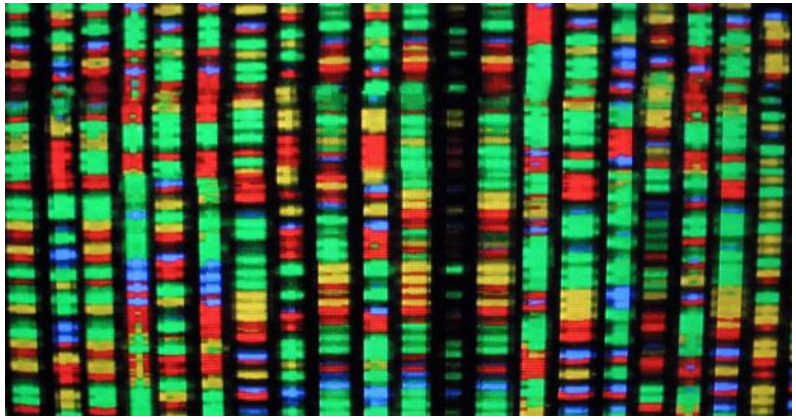
Two current models of persistence

Persistence

?

Random process

Genotypic and phenotypic features



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

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- 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

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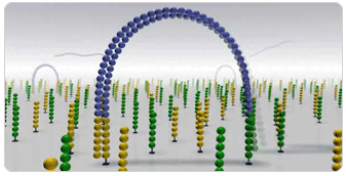
Genotype Approach - WGS

- 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

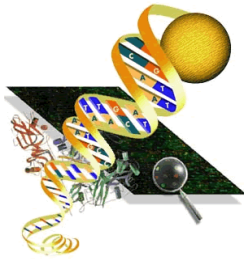
Genotype Approach – From DNA to Data

DNA → Sequencing



De novo Assembly (SPAdes)

Annotation (PROKKA)



Visualization
(Artemis and MAUVE)

Comparison: core / accessory genome (ROARY)
SNP (Parsnp)
FFP

Genotype approach- Genome Analysis

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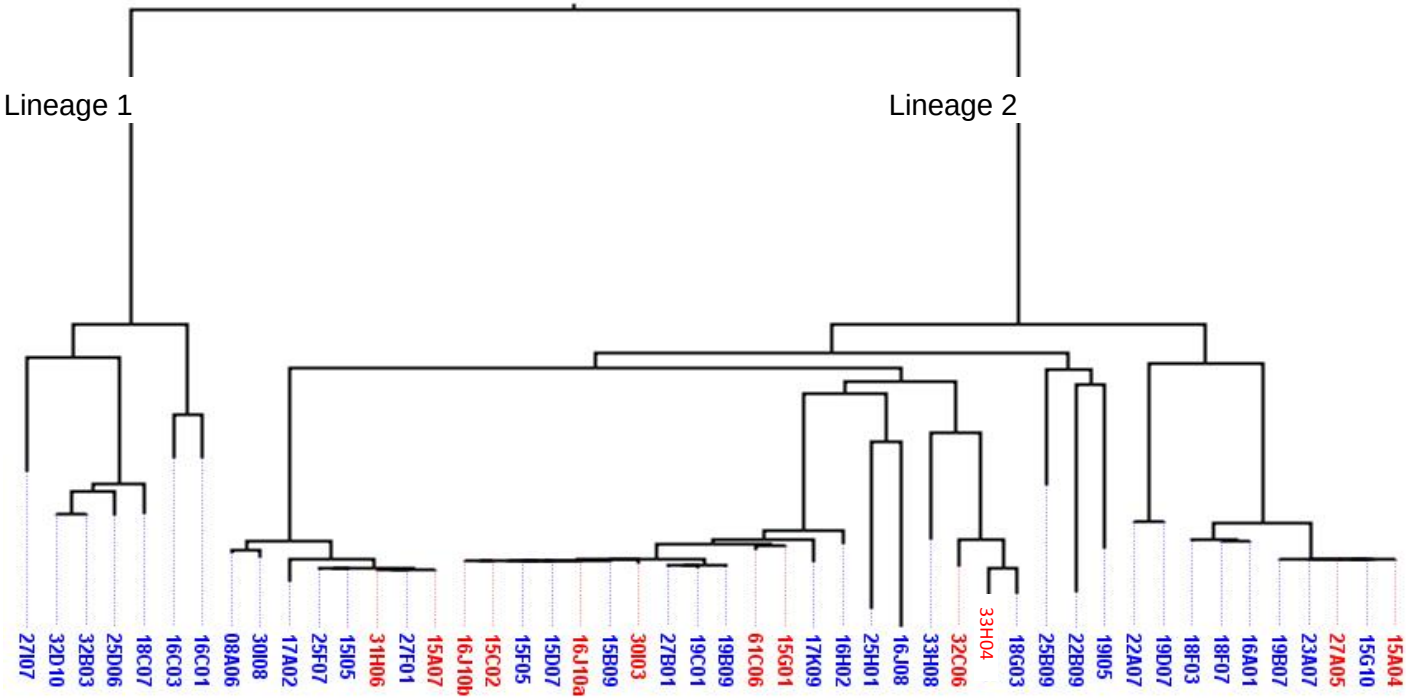


Persistent

Sporadic

→ 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

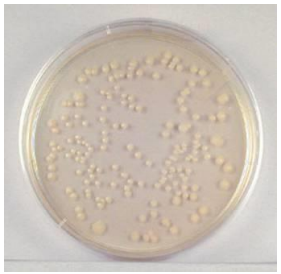
Pulsotype 3814 15A04 (plant II)
27A05 (plant I)
Pulsotype 5132 15G01 (mutant parent, plant I)
16J10 (plant I)
Pulsotype 5588 32C06 (plant III)
33H04 (plant III)
Pulsotype 6502 15A07 (plant II)
31H06 (plant II)

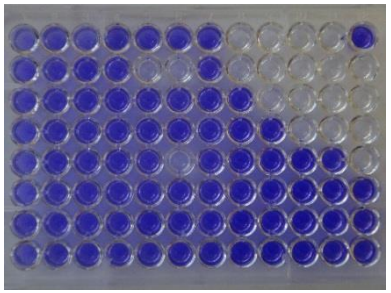
Sporadic + outbreak isolate

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

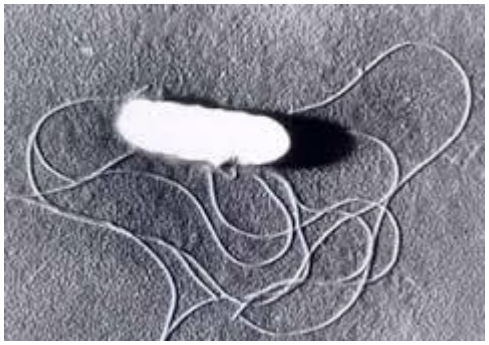
Environmental isolates

Phenotype Approach - Tests





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

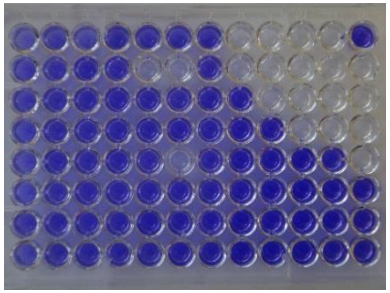


Phenotype Approach – Biofilm formation

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

Crystal violet staining

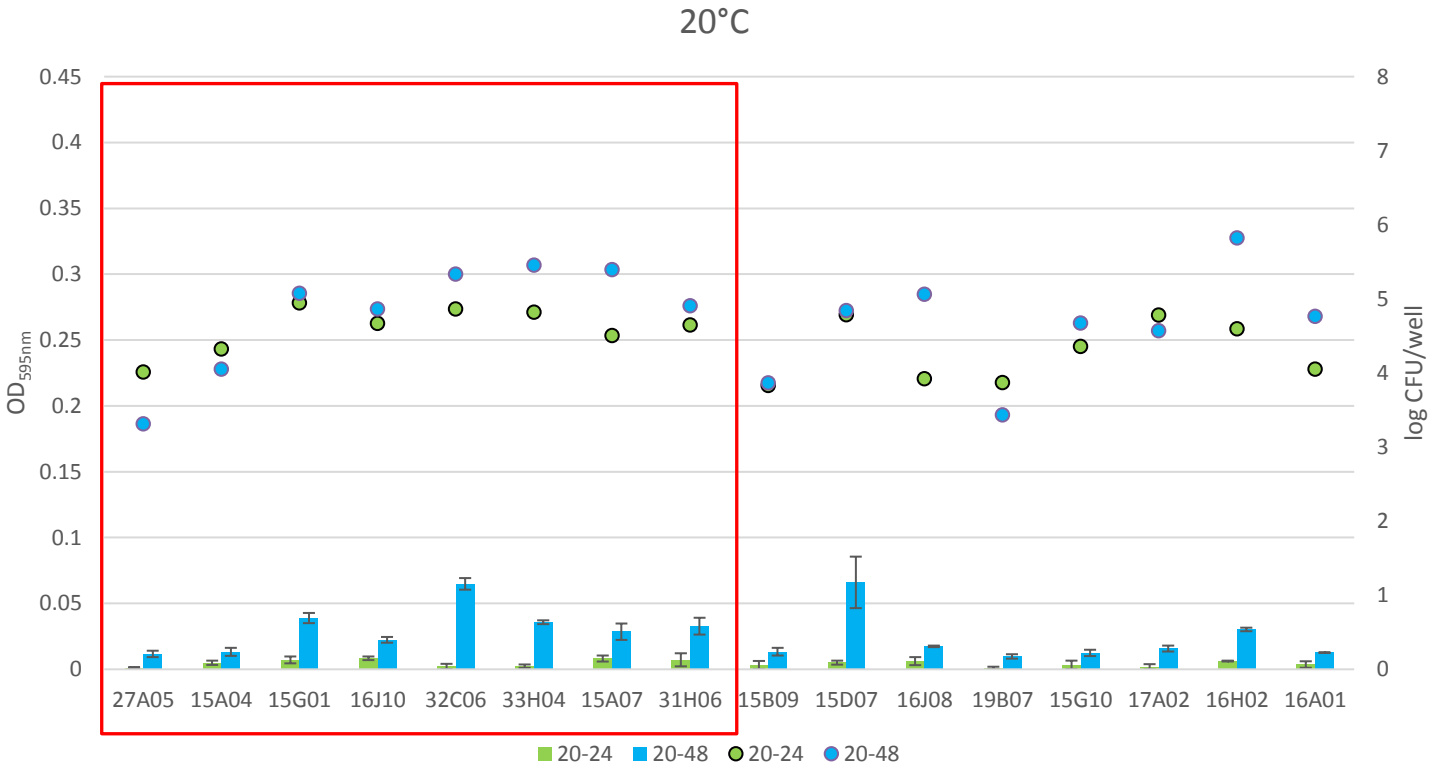
- No indication about viable cells
- Stains any organic matter



Cell enumeration by plating

- Detects viable cells
- Gives an indication about living cells in the biofilm

Phenotype Approach – Biofilm formation at 20°C

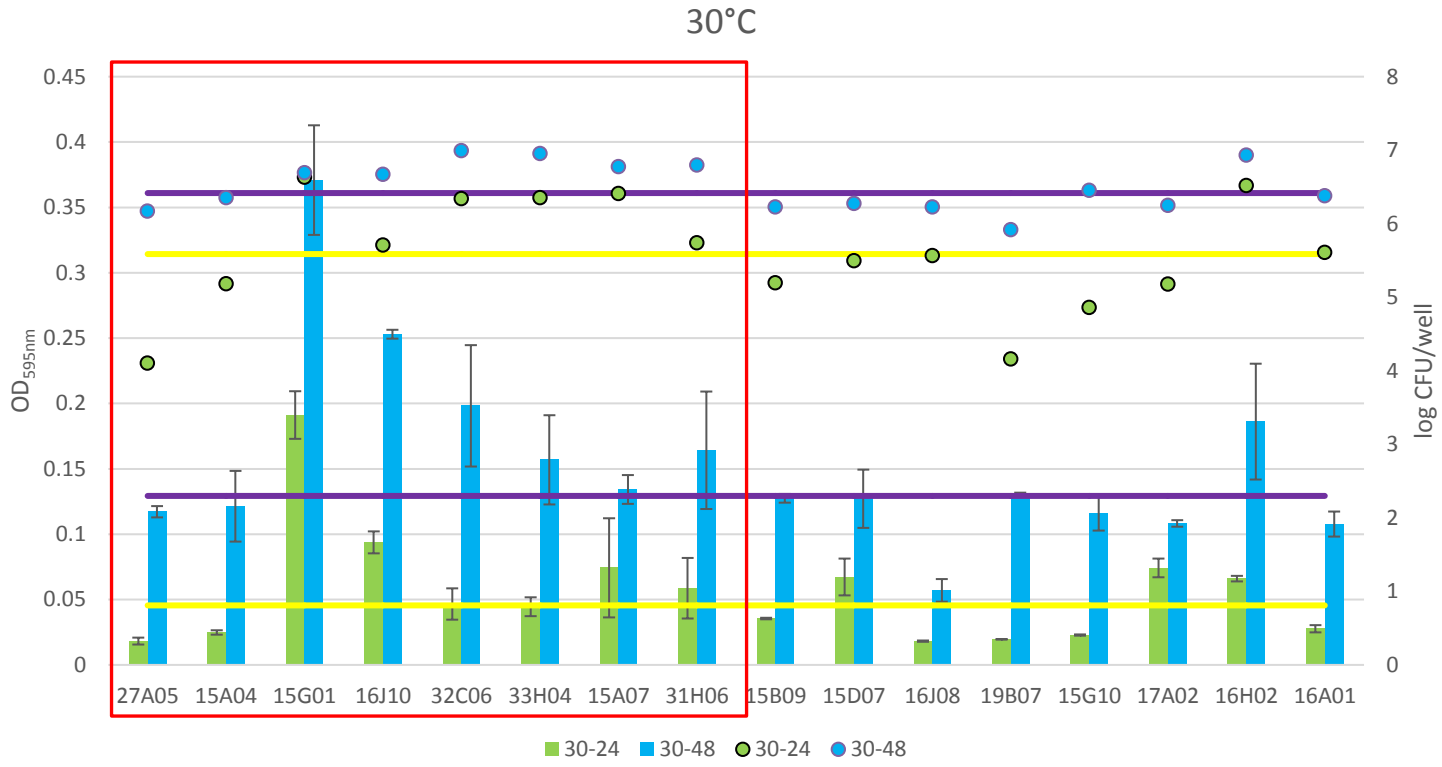


Minimal biofilm formation

Cell counts between 3-6 log CFU/well

No specific persistent behaviour

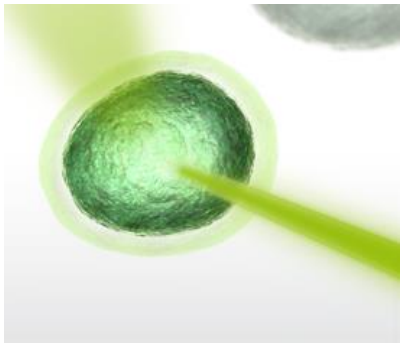
Phenotype Approach – Biofilm Formation at 30°C



- 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

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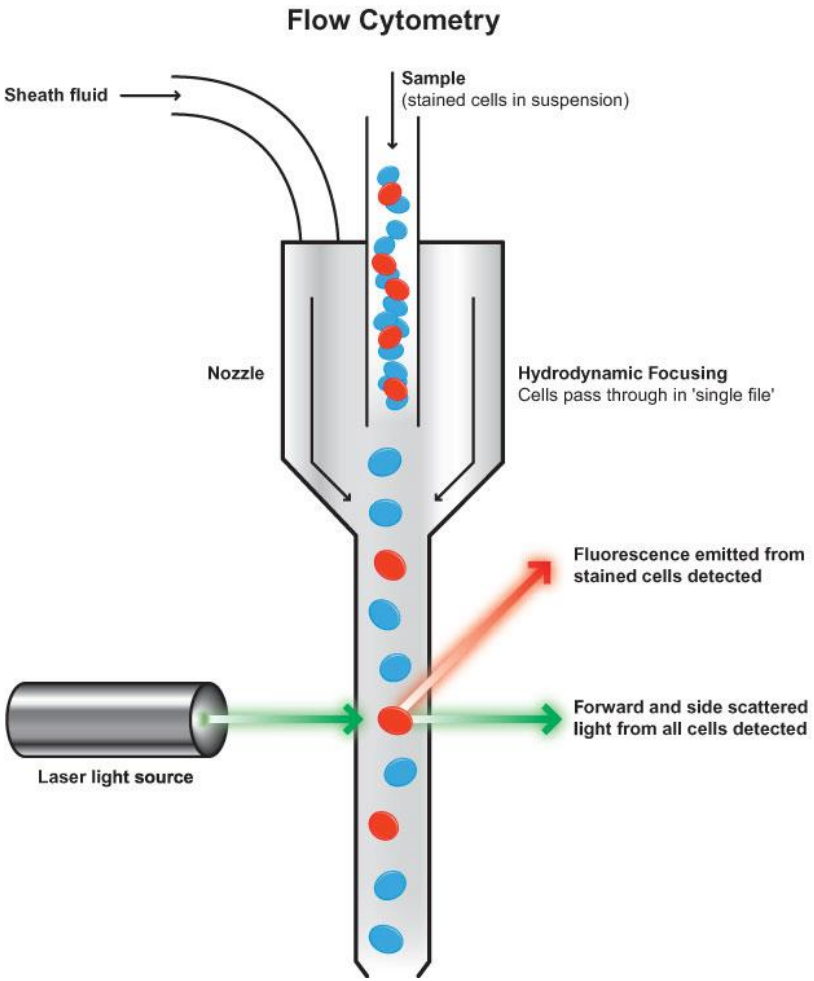

Phenotype Approach - Flow Cytometry Output

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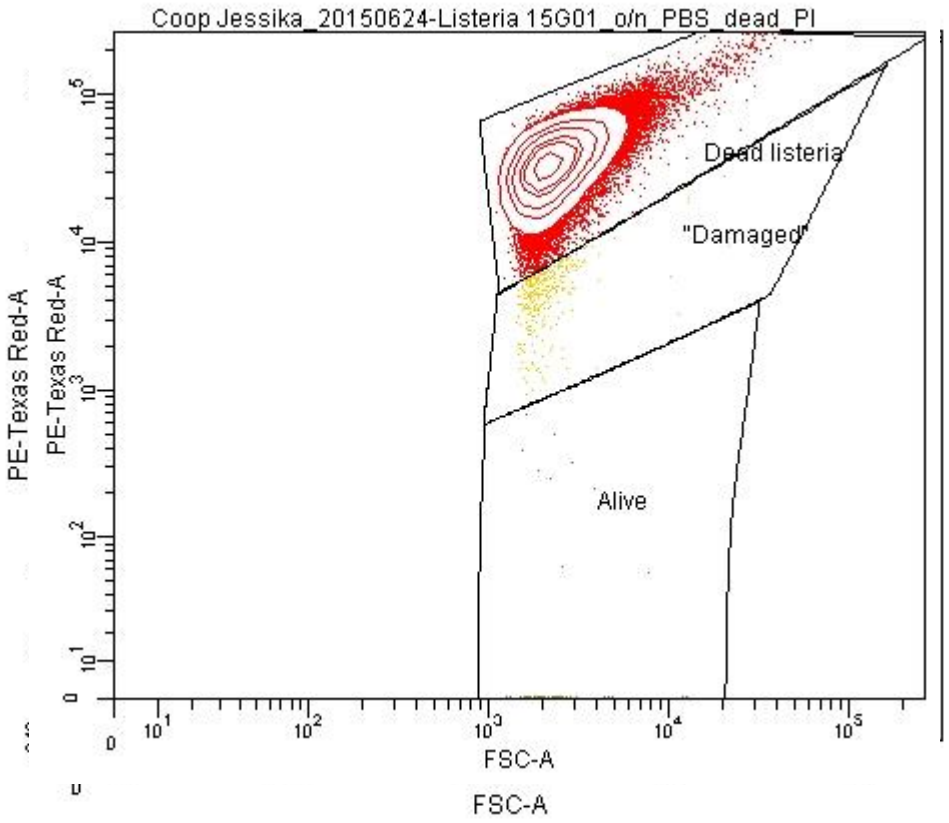

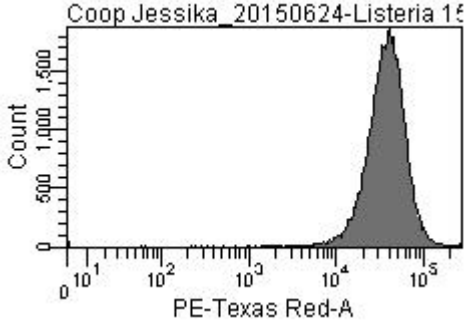

Experiment Name:	Bcereus standard PI
Specimen Name:	Coop Jessika_20150624
Tube Name:	Listeria 15G01_o/n_PBS_dead_PI
Record Date:	Jun 25, 2015 11:12:09 AM
$OP:	Administrator
GUID:	1f4aa17d-e608-4b42-8945-e318f4ba6b5b

Population	#Events	%Parent
Total cells	50,000	100.0
Dead listeria	49,650	99.3
"Damaged"	292	0.6
Alive	52	0.1

Phenotype Approach Results

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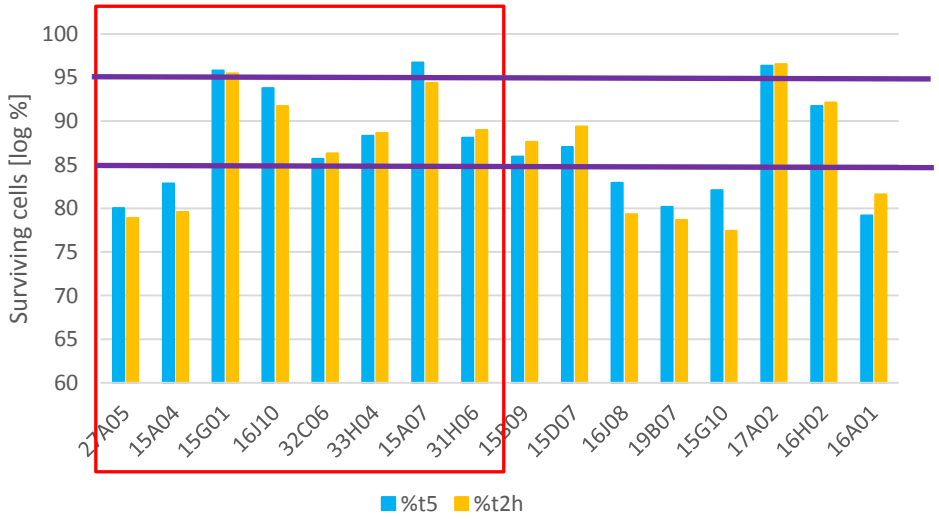
Plating



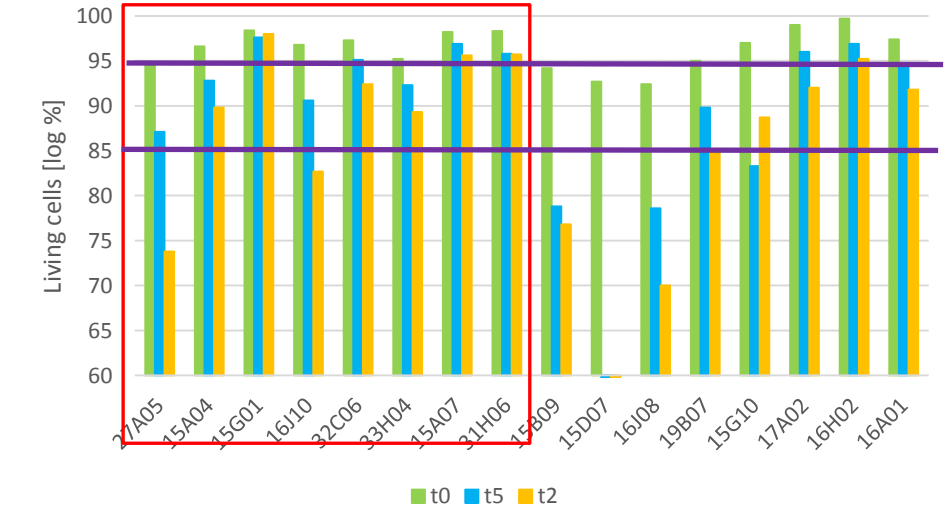
Total (persistent/sporadic)

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

	persistent	sporadic
Average t0	9.09465053	8.92693031
variance	0.01680262	0.00252948
Average t5min	8.10540189	7.65350223
variance	0.00071655	0.01332252
Average t2h	8.01439826	7.61914735
variance	0.00090569	0.00023951

Persistent/sporadic $p \leq 0.001$
Interaction $p=0.081$

Survival on dry surfaces

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Overnight culture
TSBYE
37°C

Biofilm cells

48 hours
BHI
30°C

Planktonic cells

24 hours
BHI
25°C

Incubation at 25C

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

Results

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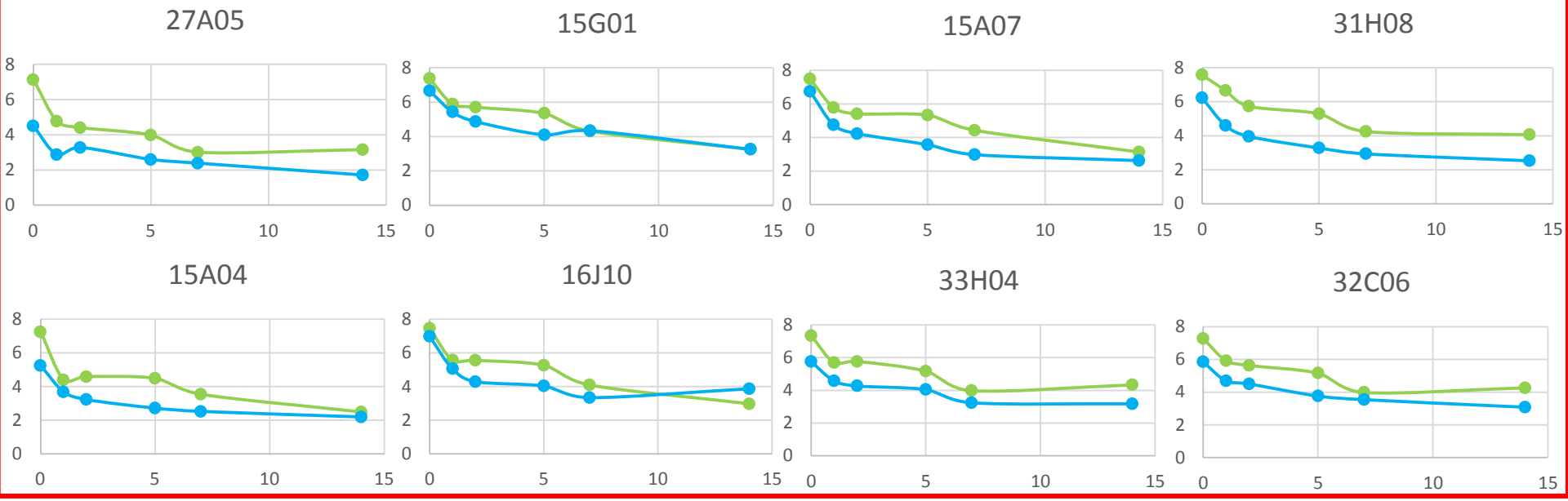

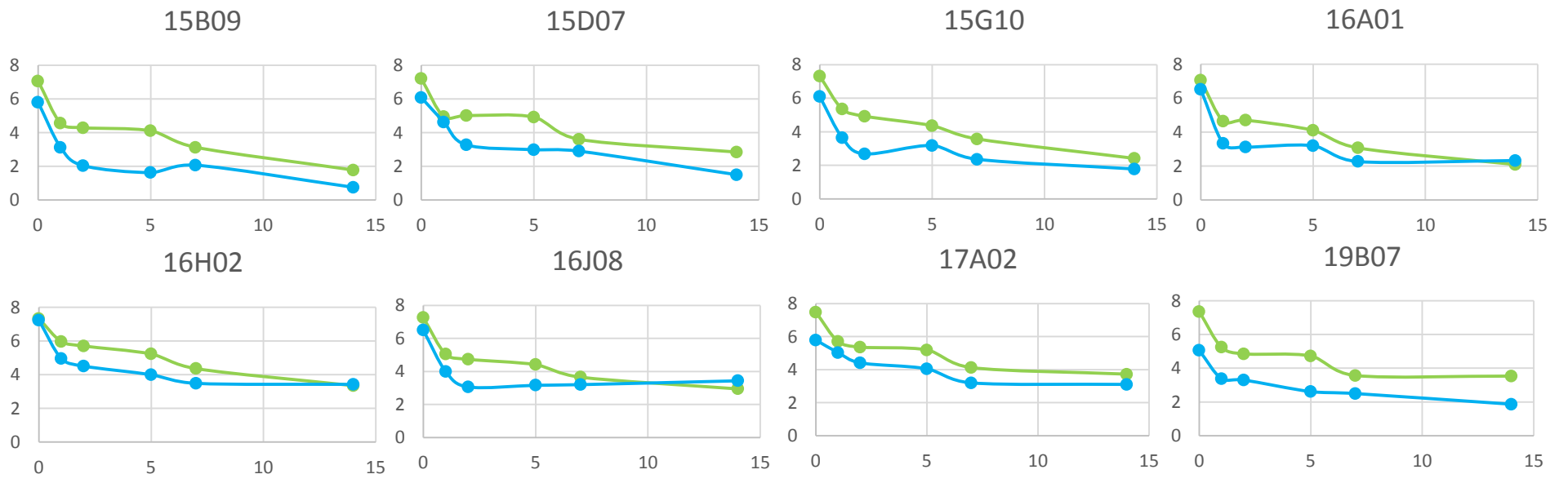

Results

- 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

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- 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)

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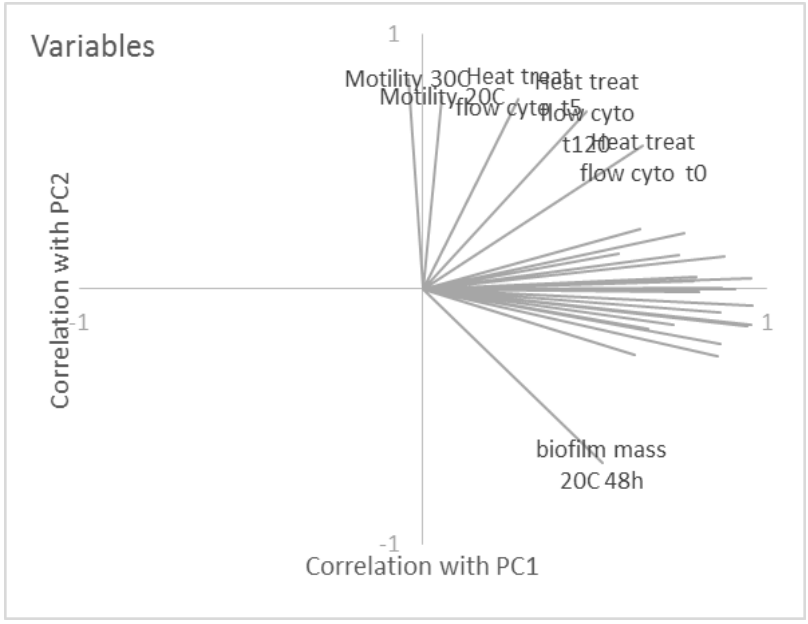

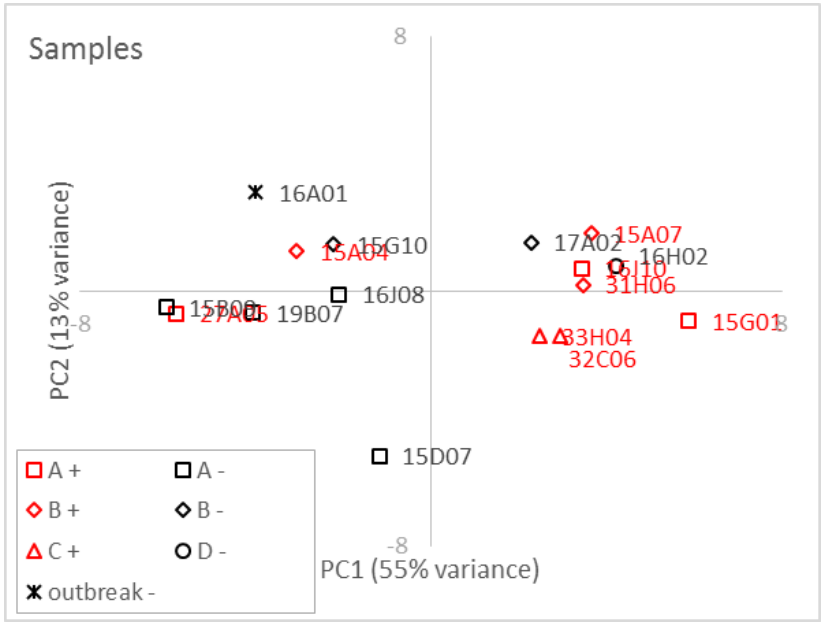

Second study
Persister cells following Nisin treatment

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- Selecting cells that survive increasing levels of nisin treatment

Gaps and limits in current studies of persister formation on food safety relevance:

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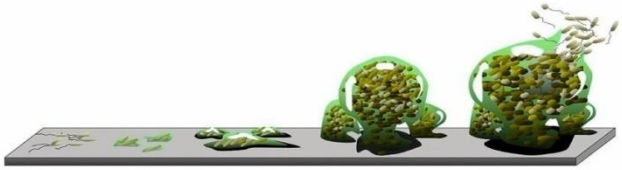
•Inadequate number persisters during sampling in food environments
Viable but Non-culturable cells – hard to detect

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

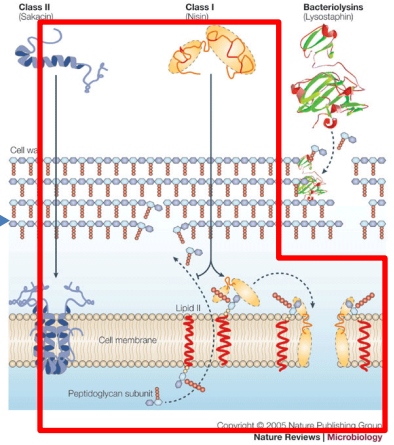
*Sanitizers： quaternary ammonium compounds, chlorine dioxide, peracetic acid



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

Product	Benefit	Application
Nisaplin DUPONT DANISCO	Anti-Gram-positive spoilage and pathogens	All types of foods: dairy, culinary, meat, bakery products and beverages

(2.5% Nisin)



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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)

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Strain	Biofilm OD 595nm		BFI	
	mean OD 595nm	standard deviation	mean BFI	standard deviation
A1	0.7	0.192	1.517	0.415
A2	0.686	0.126	1.380	0.253
A3	0.269	0.076	0.482	0.136
A4	0.324	0.052	0.715	0.115
A5	0.023	0.007	0.072	0.022
A6	0.21	0.069	0.395	0.129
A7	0.225	0.050	0.438	0.097
A8	0.101	0.017	0.184	0.031
A9	0.142	0.024	0.282	0.048
A10	0.441	0.040	0.891	0.081
A11	0.311	0.025	0.449	0.035
A12	0.226	0.043	0.438	0.083
A13	0.421	0.063	0.452	0.068
A14	0.346	0.067	0.701	0.137
A15	0.414	0.080	0.724	0.140
A16	0.118	0.033	0.236	0.065
A17	0.33	0.052	0.636	0.100
A18	0.187	0.084	0.403	0.181
A19	0.436	0.164	0.811	0.304
A20	0.097	0.008	0.198	0.017
R1	0.111	0.026	0.225	0.015
R2	0.284	0.032	0.557	0.008
R3	0.144	0.022	0.330	0.007
R4	0.129	0.022	0.311	0.012
R5	0.18	0.025	0.287	0.026
R6	0.209	0.016	0.351	0.028
R7	0.096	0.006	0.163	0.030
R8	0.143	0.019	0.351	0.010
R9	0.199	0.021	0.302	0.024
M1	0.193	0.053	0.338	0.097
M2	0.147	0.012	0.377	0.031
M3	0.173	0.052	0.300	0.094
M4	0.235	0.042	0.441	0.090
M5	0.668	0.106	1.537	0.257
M6	0.072	0.013	0.155	0.025
M7	0.11	0.014	0.329	0.047
H1	0.101	0.018	0.158	0.029
H2	0.155	0.039	0.241	0.061
H3	0.089	0.015	0.151	0.025
H4	0.106	0.014	0.172	0.022
H5	0.128	0.029	0.200	0.045
H6	0.081	0.023	0.130	0.037
H7	0.112	0.023	0.172	0.035
H8	0.135	0.009	0.209	0.014
H9	0.085	0.012	0.145	0.020
H10	0.121	0.025	0.193	0.040
H11	0.112	0.027	0.173	0.041
H12	0.101	0.014	0.160	0.023

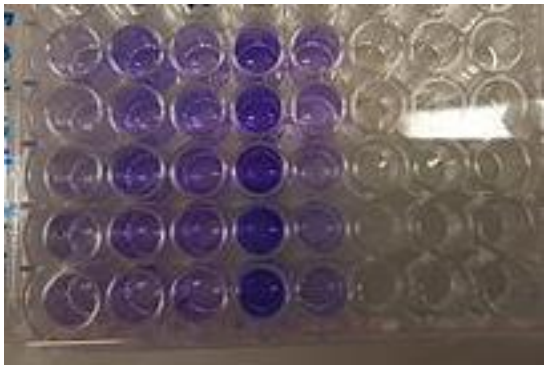
A1-20: AsureQuality Limited, NZ;

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):

BFI = (AB-CW)/G

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

strong (≥1.10),
moderate (0.70–1.09),
weak (0.35–0.69)

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

Identify the presence of *L. monocytogenes* persister cells by dose-dependent killing of planktonic cells

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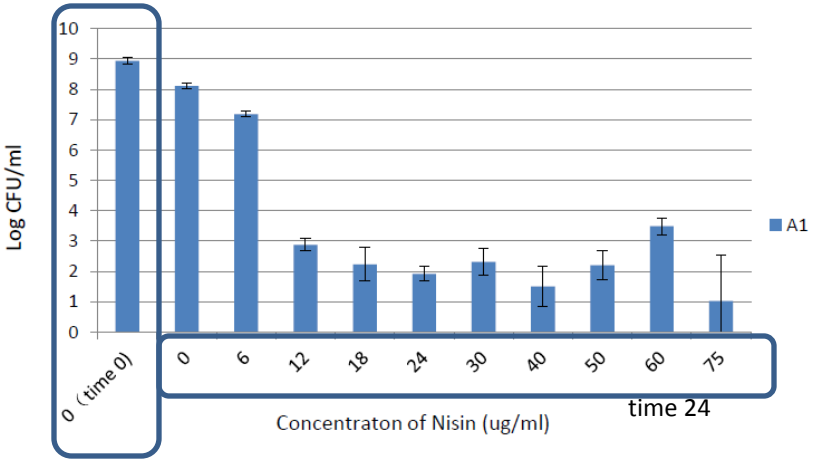
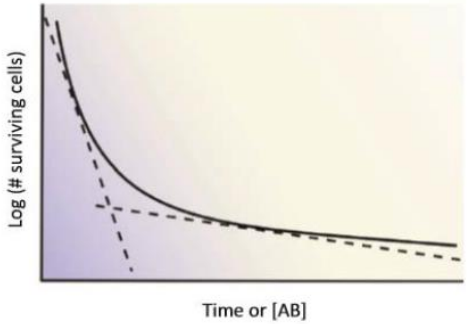


100µl blank/nisin + 900µl overnight culture



under spent medium environment

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.

antibiotic concentration ([AB]) is x-axis.

•Tolerant to prolonged treatment with high dosed of bactericidal nisin;

•Genetically identical to susceptible bacteria;

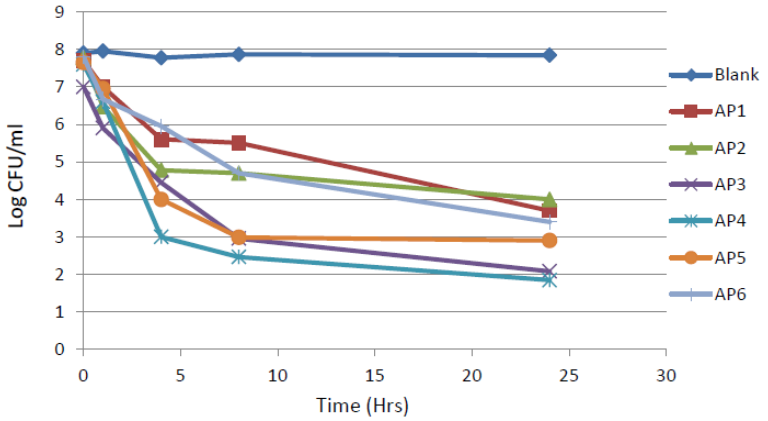


Figure 3b. Six persister isolates from theA1 strain (AP1-AP6) which survived 24hrstreatment with 75µg/ml nisin in TSB were re-exposed to 75µg/ml nisin at 30°C for 24hrs.

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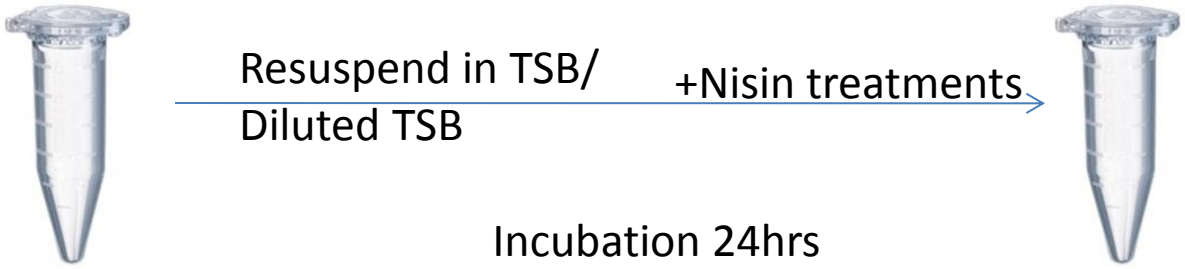
What if we resuspended overnight culture cells in to new medium ?

and

How would the resuspended cells respond to nisin treatment?



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

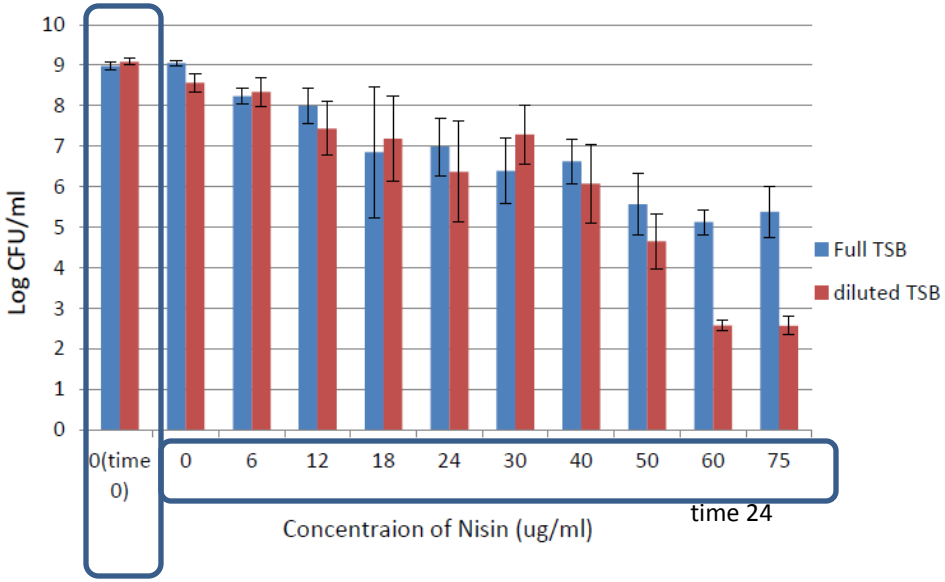
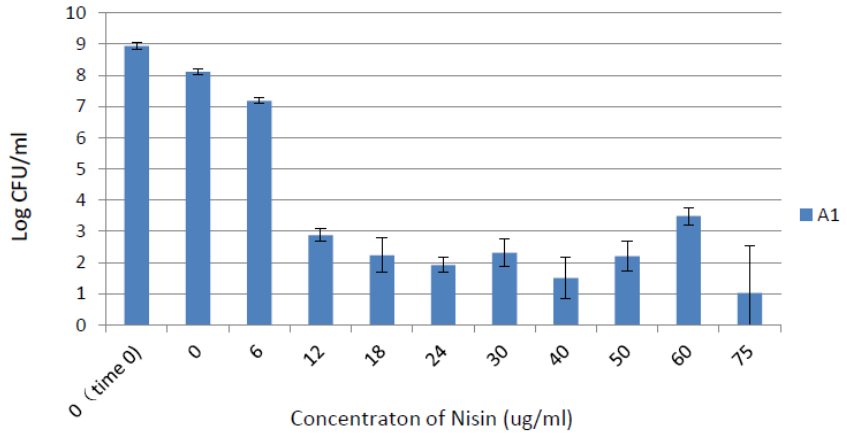


Figure 4a Dose-dependent killing of re-suspended cells of the A1 strain. The blue bars represent an

The re-suspension cells showed increased persistence;

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



Nutrient limitation?

Whether limited nutrient condition favours persister formation?

Resuspend in TSB/
Diluted TSB

Incubation first 24hrs

time 0

+Nisin treatments

Incubation second 24hrs

time 24

4 The effect of nutrients on the production of *L. monocytogenes* persister cells

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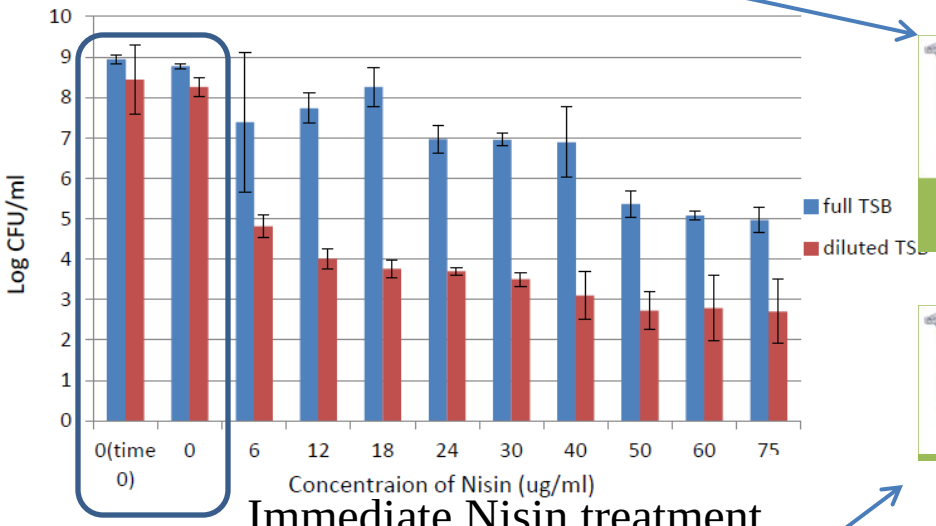
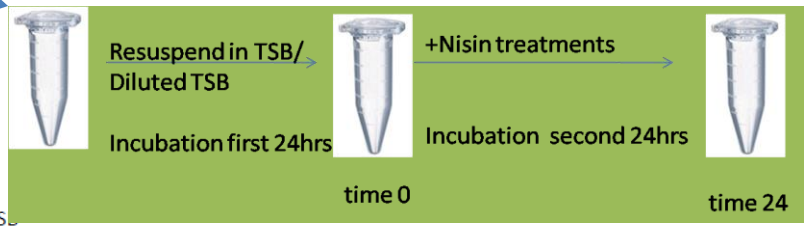
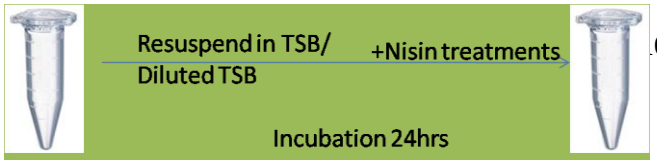
Delayed Nisin treatment







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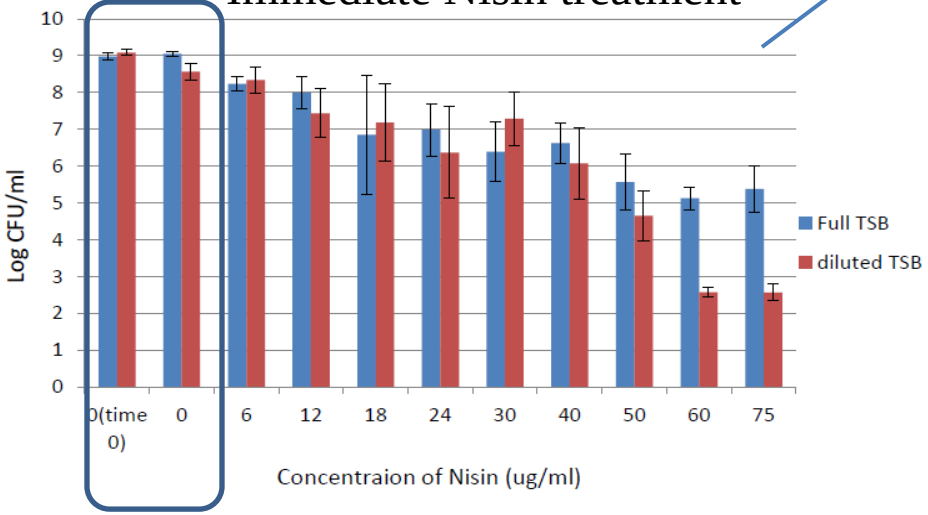
Immediate Nisin treatment



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

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What about persisters in biofilm following with nisin treatment?

Optimizing methods for obtaining *L. monocytogenes* persisters in a biofilm model

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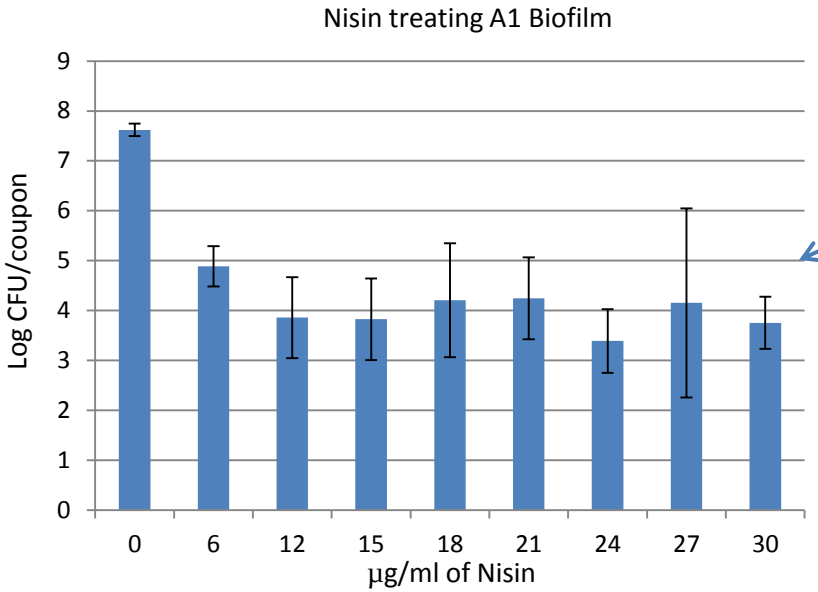
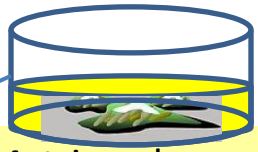
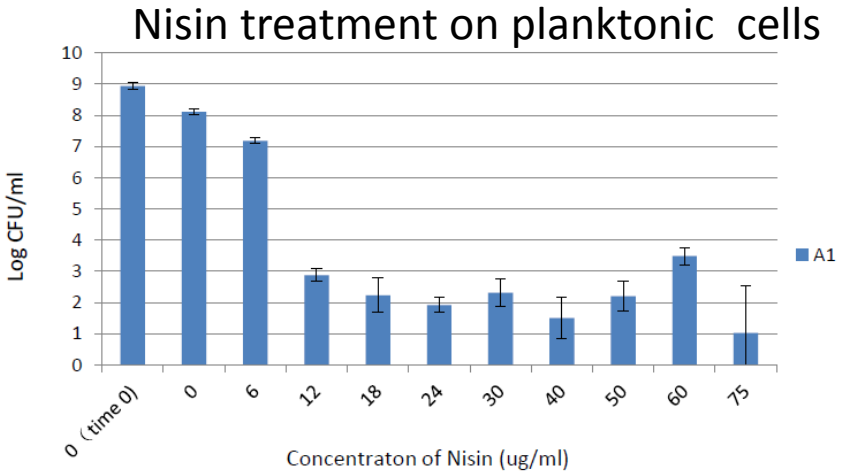




Matrix polymers

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)**

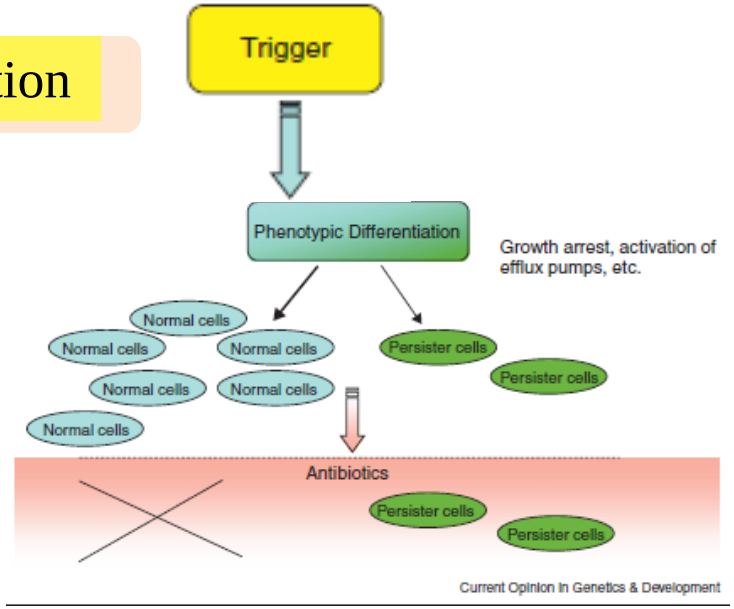
-*L. monocytogenes* persister formation is influenced by specific features in a biofilm community **(e.g. structure of the extracelullar polymers)**

- *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

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- 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

Gene name	Function	Increase/decrease
lmo1580	Universal stress protein	+ 2.89
lmo2004	Transcription regulator	- 4.91

Select gene expression chnages

- Cell wall synthesis

Gene name	Function	Increase/decrease
lmo0129	amidase	+4.09
lmo2714	Peptidoglycan bound protein	-3.44

Select gene expression changes

- DNA repair and damage

Gene name	Function	Increase/decrease
lmo1975	DNA polymerase IV	-4.03

- No genes upregulated

Select gene expression changes

- ATP binding /transport system

Gene name	Function	Increase/decrease
lmo1636	ATP-binding protein	+3.58
lmo1730	Sugar transport	-3.49

Select gene expression changes

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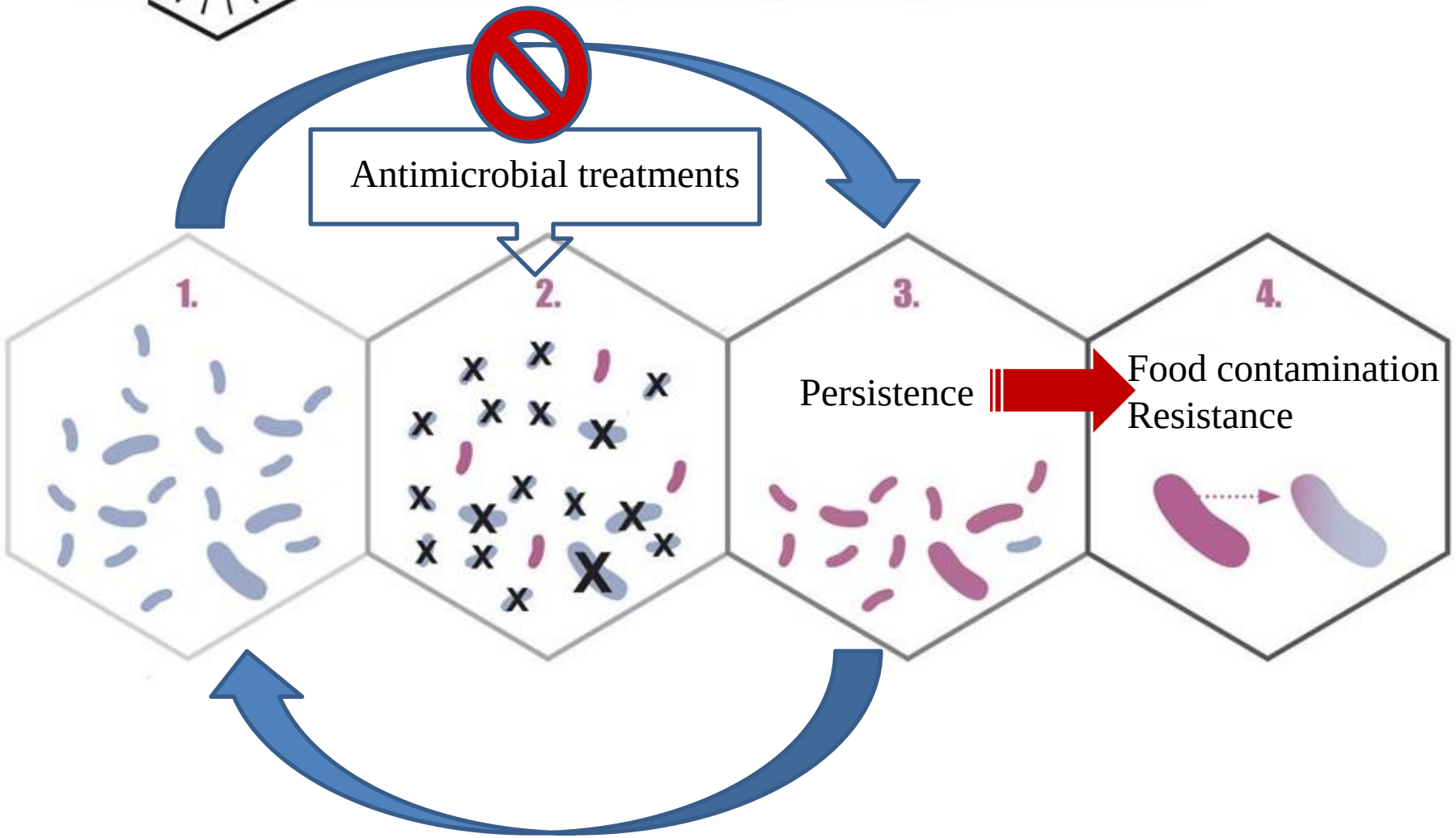
- Bacteria change their gene expression to cope with preservatives/sanitisers
- Suggests going into “lock down” or “sleep” until conditions improve
- A natural temporary protective mechanism
- Does this “evolve” into resistance?

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

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