The role of bacterial dormancy in their persistence and survival in the food chain

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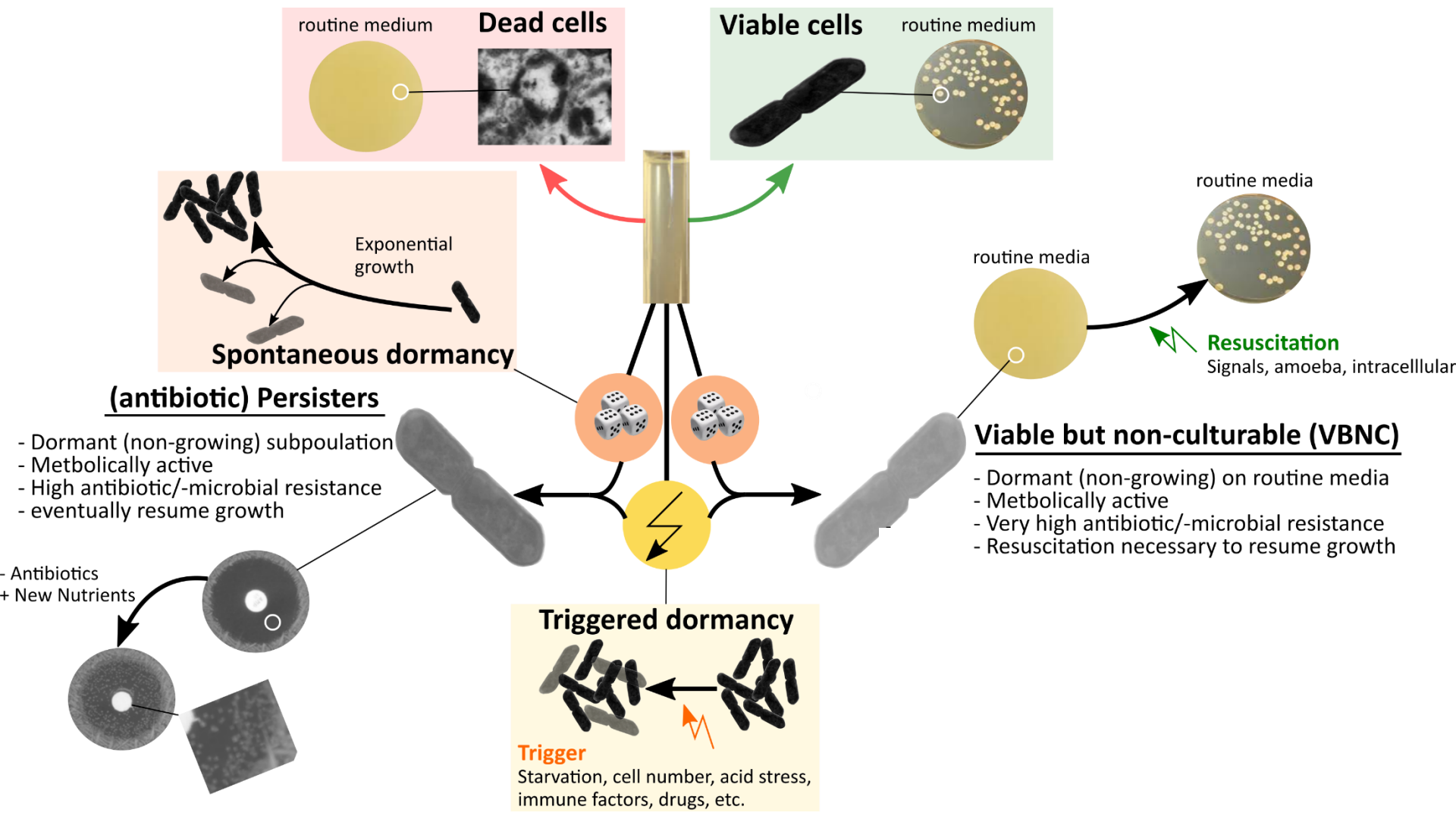
Talk outline

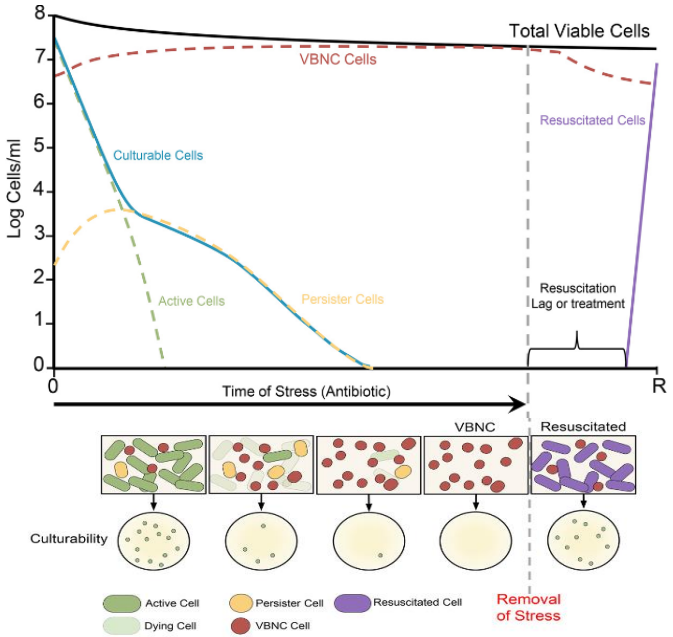
- Bacterial dormancy
  - Explore different types of dormancy
  - Possible connections and challenges
- Dormancy and Food production
  - What do we know
  - What does it mean (for food safety)
- Where should go next

The power of doing nothing


routine medium
Dead cells
Viable cells
routine medium
Exponential growth
Spontaneous dormancy
routine media
routine media
Resuscitation
Signals, amoeba, intracelllular
(antibiotic) Persisters
- Dormant (non-growing) subpoulation
- Metbolically active
- High antibiotic/-microbial resistance
- eventually resume growth
Viable but non-culturable (VBNC)
- Dormant (non-growing) on routine media
- Metbolically active
- Very high antibiotic/-microbial resistance
- Resuscitation necessary to resume growth
- Antibiotics
+ New Nutrients
Triggered dormancy
Trigger
Starvation, cell number, acid stress, immune factors, drugs, etc.

Definitions

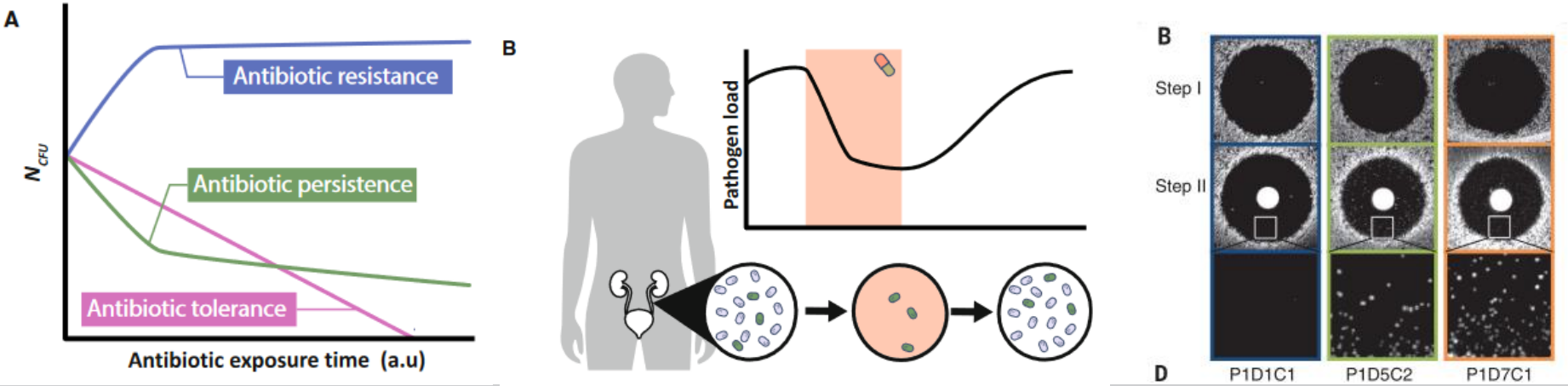
- Proliferating
  - Proliferating (culturable) cells
- Stressed/injured
  - Sublethal (reparable) injury in a proportion of the population
  - Not/less culturable on selective media
- Persistent
  - Dormant (non-growing) subset present in the population
  - Culturable on routine media
- **V**iable **b**ut **n**on-**c**ulturable (VBNC)
  - Dormant (non-growing) subset present in the population
  - Non-Culturable on routine media
- Dead
  - Metabolically inactive or disintegrated cells



Ayrapetyan et al. (2018) doi: 10.1128/JB.00249-18.

Bacterial dormancy - Persister

- Antibiotic persistence is **a survival strategy where only a small subpopulation is highly tolerant to the antibiotic**.
- This results in characteristic biphasic killing, where the majority of sensitive cells are rapidly killed and the subpopulation of persister cells survives.



Bollen et. al EMBO reports 24: e57309 | 202 *DOI 10.15252/embr.202357309*
Liu et al., Science 367, 200–204
Staples et al.; Salmonella persisters undermine host immune defenses during antibiotic treatment; Science 362, 1156–1160

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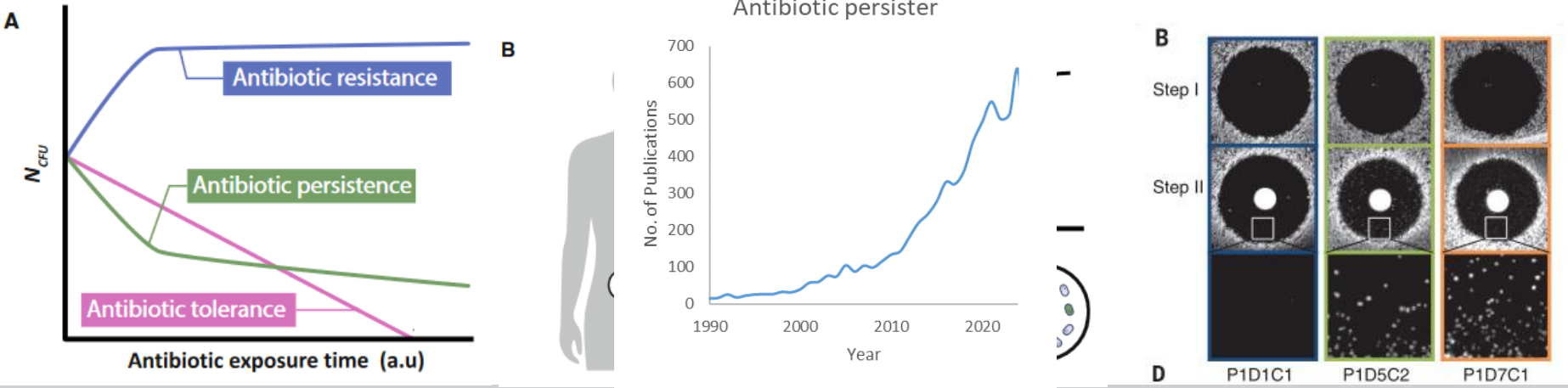

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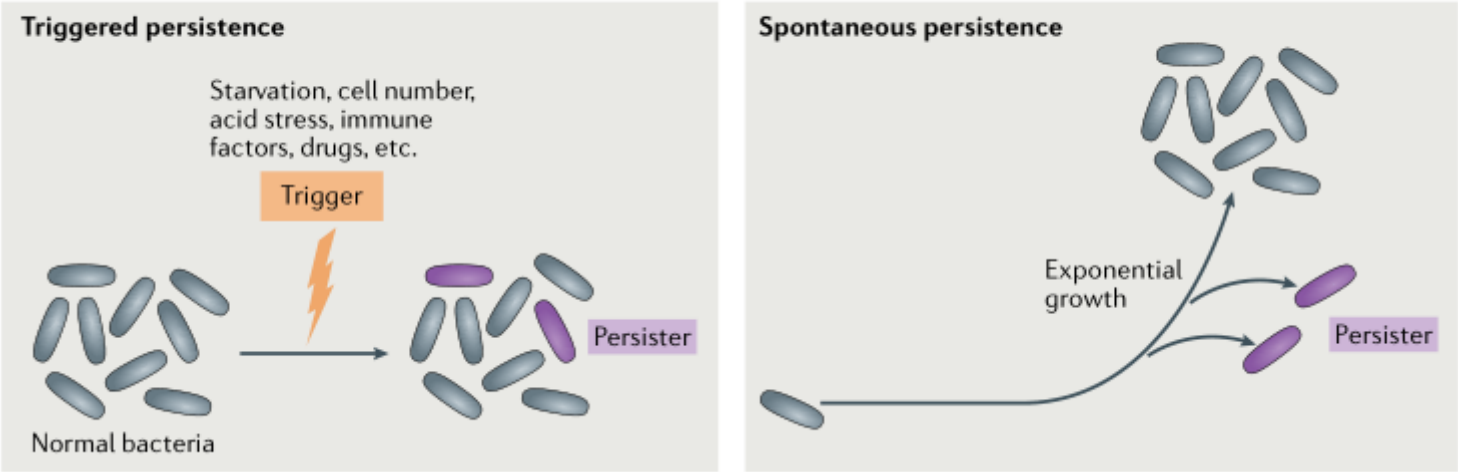
Bacterial dormancy - Persister

- Induction of persistence



Balaban et al.; Definitions and guidelines for research on antibiotic persistence; Nat Rev Microbiol 17, 441–448 (2019).
https://doi.org/10.1038/s41579-019-0196-3

Bacterial dormancy - Persister

- **Passive defense of persister cells against antibiotic**

➔ **Entering a dormant state**

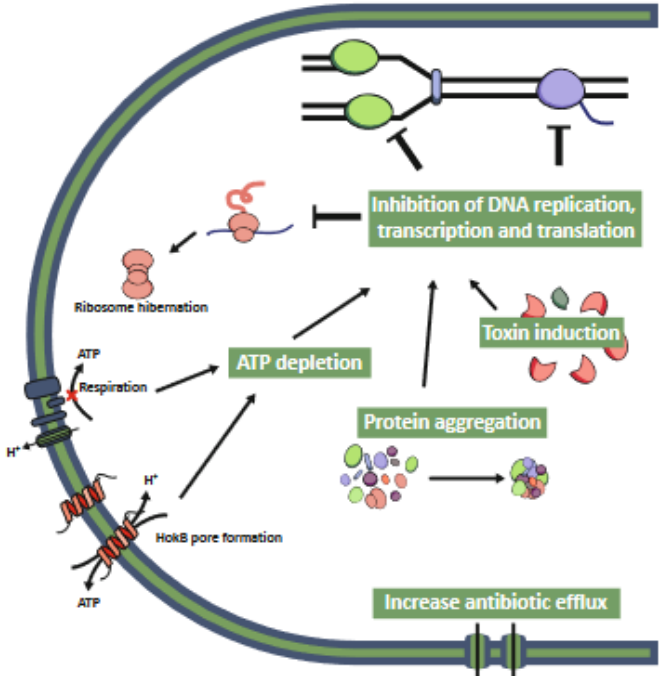
- **Active defense of persister cells against antibiotics**
  - increasing efflux activity
  - preventing prodrug activation
  - activating stress responses

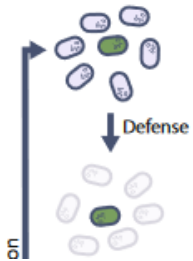
Bacterial dormancy - Persister

- **Passive defense of persister cells against antibiotic**

➔ **Entering a dormant state**

- ATP depletion
- Inhibition of DNA replication
- Toxin induction
- Protein aggregation





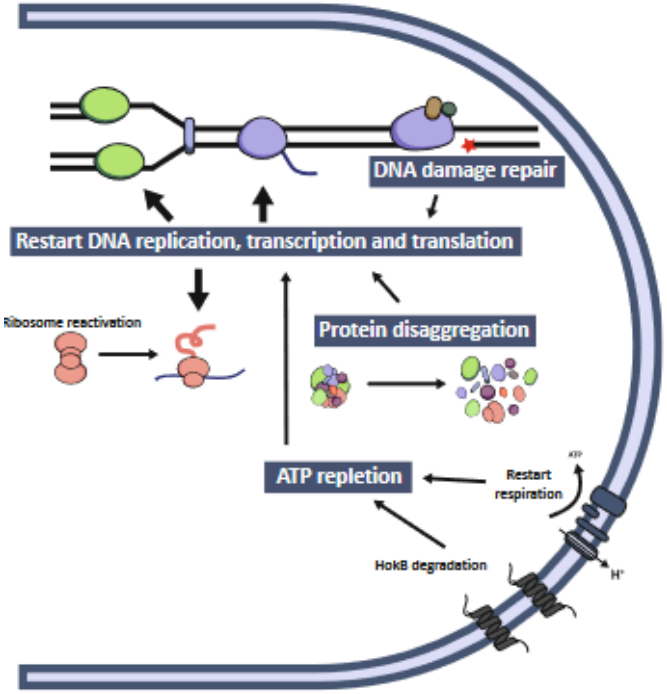
Bollen et. al EMBO reports 24: e57309 | 202 *DOI 10.15252/embr.202357309*

Bacterial dormancy - Persister

- **Passive defense of persister cells against antibiotic**

➔ **Leaving a dormant state**

- fresh nutrients
- quorum sensing signals from growing cells
- removal of certain host immune factors



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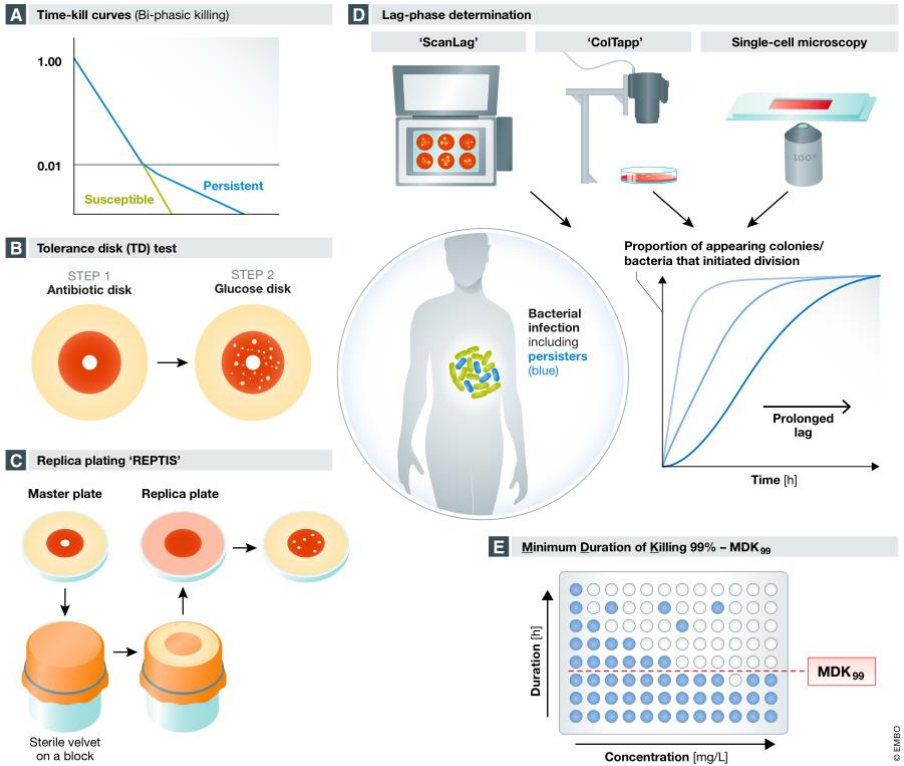
Bacterial dormancy - Persister

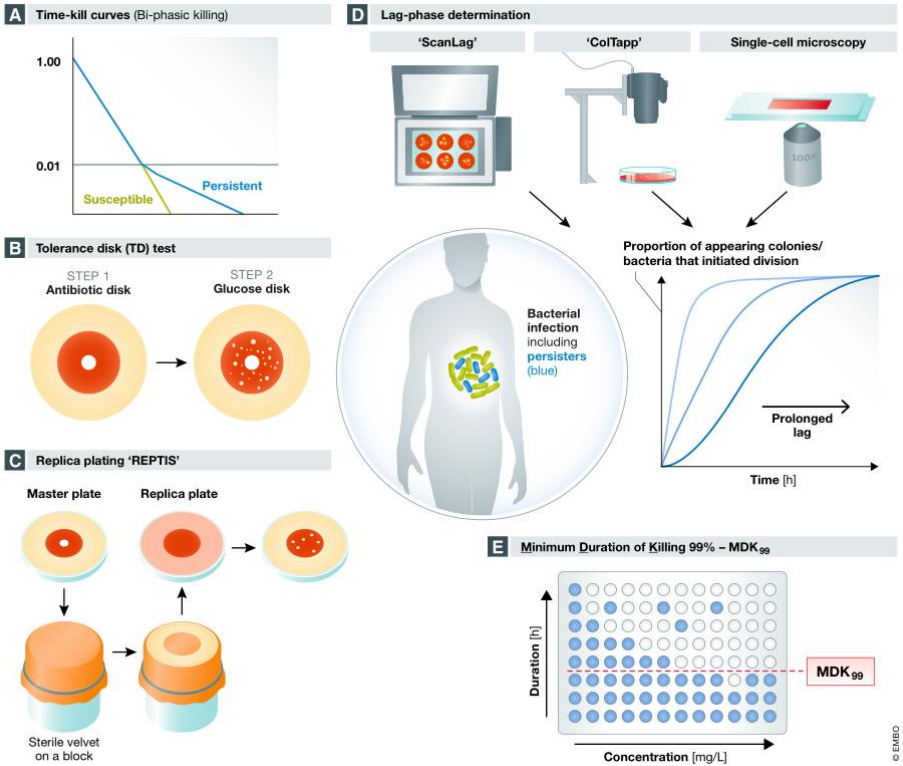


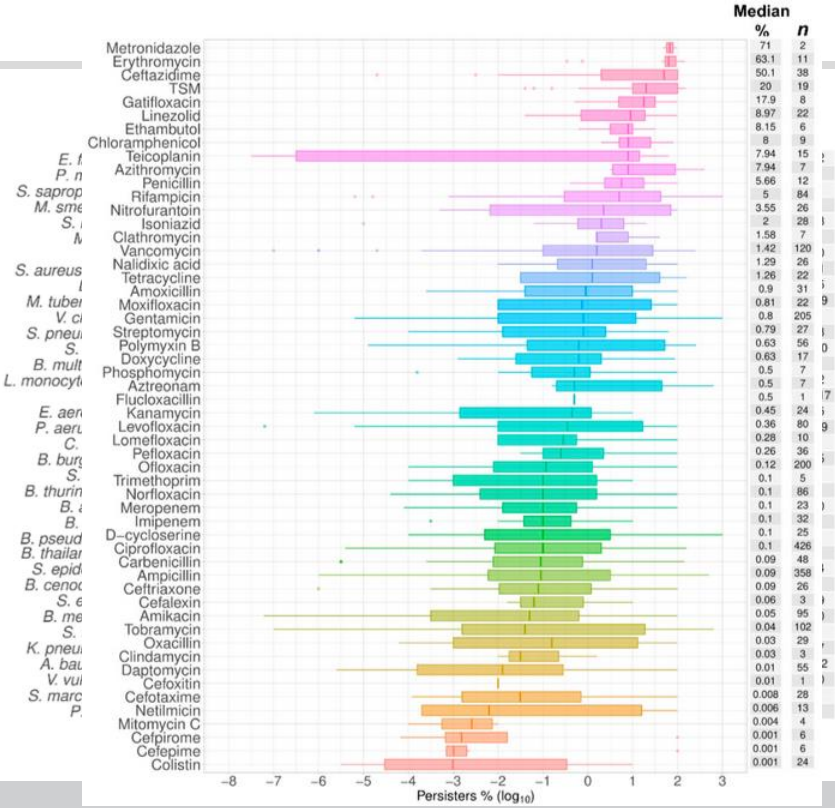
- Quantify and study a **small tolerant subpopulation** of dormant cells within a **culturable population**

➔ **Kill the susceptible** part of population and study the survivors

Salcedo-Sora; Antibiotics 2020, 9, 508;
doi:10.3390/antibiotics9080508

Bacterial dormancy - Persister





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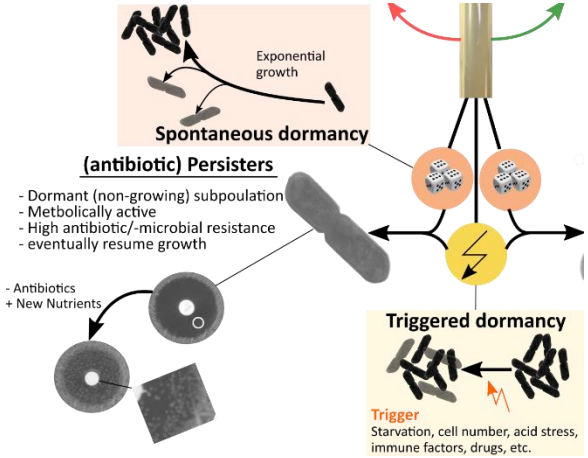
Bacterial dormancy - Persister



- Antibiotic persistence is **a universal survival strategy** where only a small subpopulation is highly tolerant
- The passive defense of persisters is reliant on cells entering a **dormant state**
- Cells leave the dormant state by **replenishing** their energy level and **reactivating** crucial cellular pathways
- It's an **umbrella term** describing different physiological states

➔ There is an existing consensus paper **clearly defining** persistence

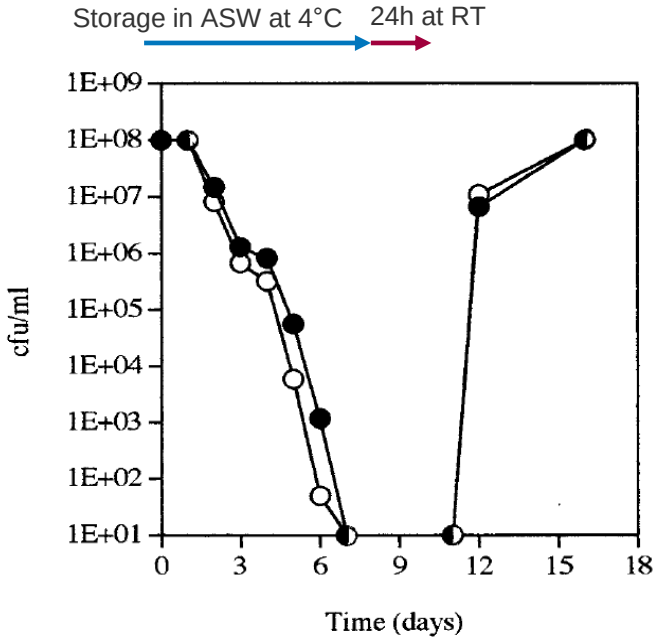
➔ On this basis methods / approaches can be **evaluated and tested**

Bacterial dormancy - VBNC

Bacterial dormancy - VBNC

- *Vibrio Vulnificus*
  - Pathogenic bacteria present in marine environments
  - inability to culture *V. vulnificus* during winter or cold months
  - Storage in artificial sea water (ASW) at 4°C induces entry into the VBNC state
  - Stable (and quantitative) resuscitation from the VBNC state by storage at RT for one day before plating
  - Model system for VBNC

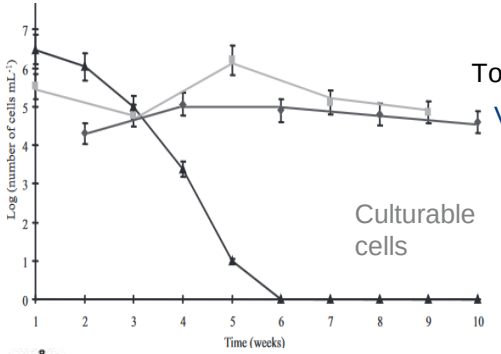
A bacterial cell in the VBNC state may be defined as one which **fails to grow at the routine bacteriological cultivation conditions under which it would normally grow**, but which is in fact alive and has still metabolic activity.



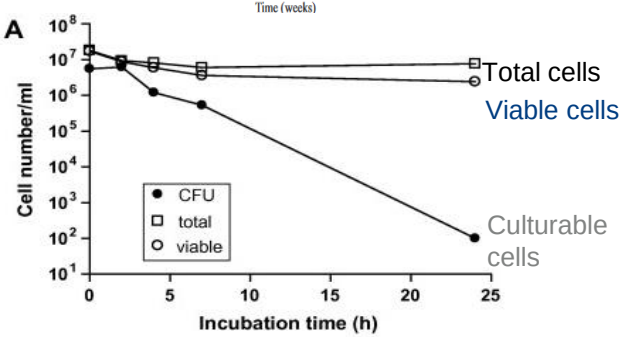
Oliver et al. 1995; doi: 10.1111/j.1574-6968.1995.tb07885.x.

Bacterial dormancy - VBNC

- Subset of any bacterial population is in the VBNC state
- Entry into VBNC state is a stress response
  - Slow induction (days to years)
    - Response to unfavorable environmental conditions
    - Shifts of temperature, osmotic pressure, a_w value or starvation, pH, sunlight



7% NaCl
20°C

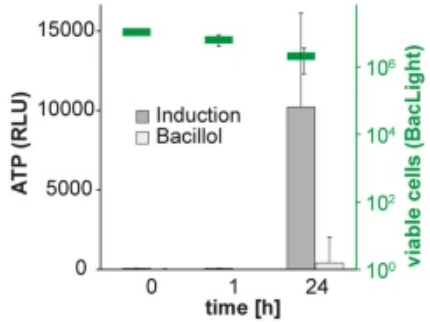


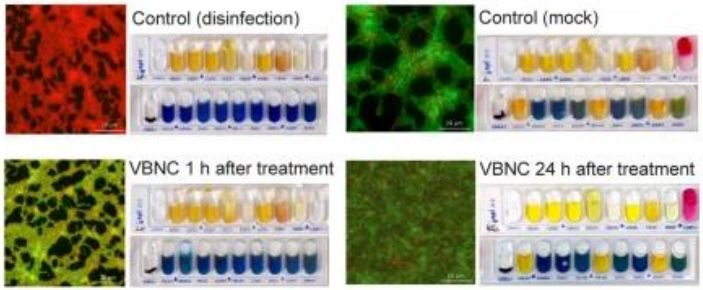
pH 4
37°C

Besnard 2002; DOI: 10.1051/vetres:2002022
Cunningham et al. 2009;
doi:10.1016/j.foodcont.2009.03.005

Bacterial dormancy - VBNC

- Subset of any bacterial population is in the VBNC state
- Entry into VBNC state is a stress response
  - Slow induction (days to years)
    - Response to unfavorable environmental conditions
    - Shifts of temperature, osmotic pressure, a_w value or starvation, pH, sunlight
  - Fast induction (<60 min)
    - Response to potentially lethal stress
    - Heat shock, UV, biocides, chlorine, PAA, oxidizing agents, heavy metals etc.
    - Benzalkonium chloride; non-ionic detergents and salts





Robben et al. 2018; doi: 10.1038/s41598-018-33595-5

Defining bacterial viability

Viability Parameter

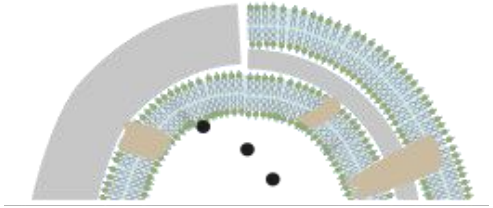
Cell proliferation

Cell integrity

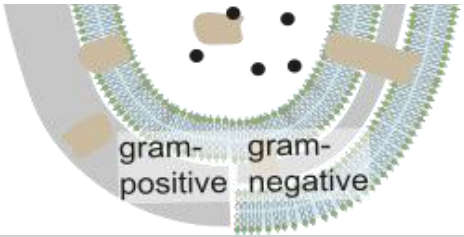
Prevent loss of cellula content (e.g. genetic material; ribosomes e

Active metabolism

Retain the ability for resuscitation



To be considered alive, a cell "must be ***intact***, ***capable of reproduction***, and ***metabolically active***"



- Quantify and study a **small tolerant subpopulation** of dormant cells within a population containing **culturable and dead cells**

➔ Differentiate viable from dead cells

How to measure VBNC

Viability Parameter

and what is actually measured

Cell proliferation

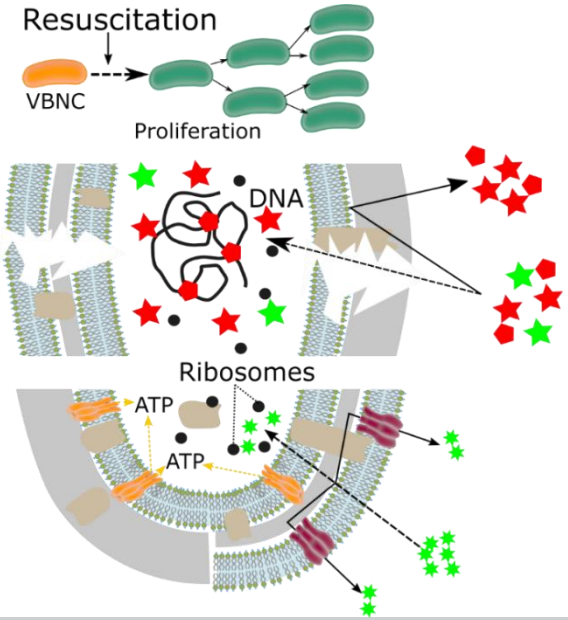
Cell growth; e.g.
Colony forming units

Cell membrane integrity

Exclusion of molecules from intact membranes

Active metabolism

Production of cellular energy; Efflux pump activity; Transcription; Translation; Enzyme activity



How do we measure?

Quantitative resuscitation; cell elongation

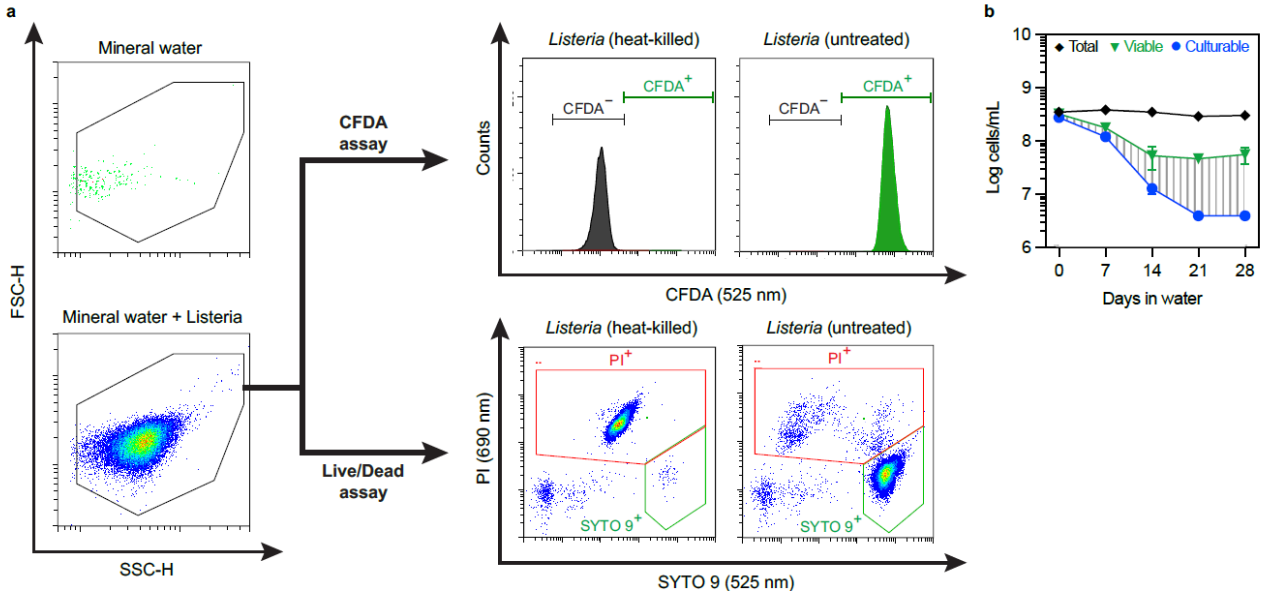
Fluorescence stains (Microscopy or FACS); PMA/EMA-qPCR; electron microscopy

De novo ATP production; fluorescence stains (FACS); RT-qPCR; protein labelling; GFP expression; fluorescence labelled substrates

Fleischmann et al. 2021, https://doi.org/10.3390/antibiotics10020115

How to measure VBNC

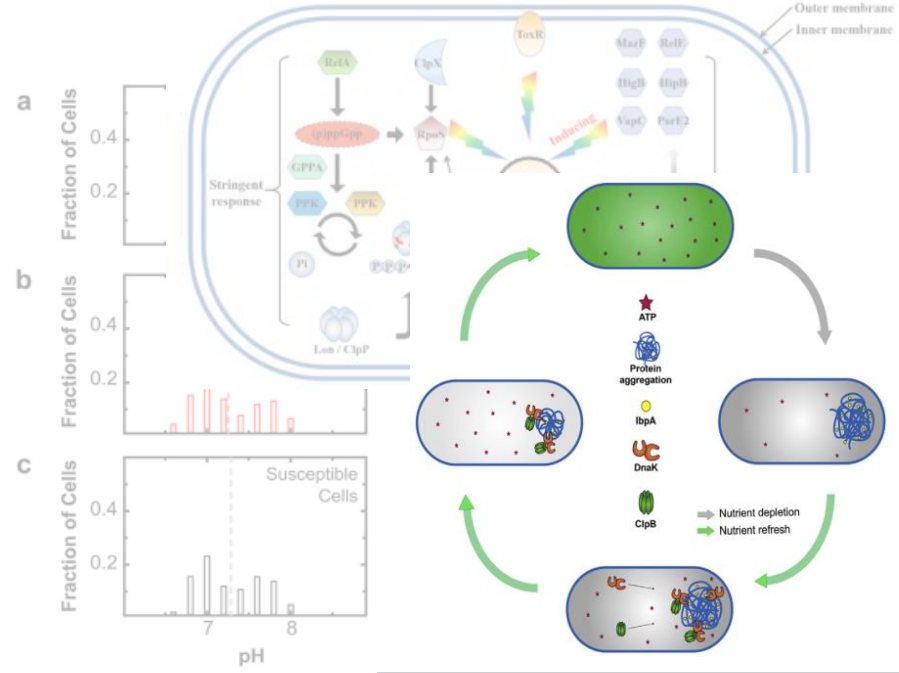
- It is crucial to use **at least two different** viability parameters for VBNC detection /quantification
- There is no official consensus



Carvalho et al. 2024; Nature Communications; doi:10.1038/s41467-024-52633-7

Bacterial dormancy - VBNC

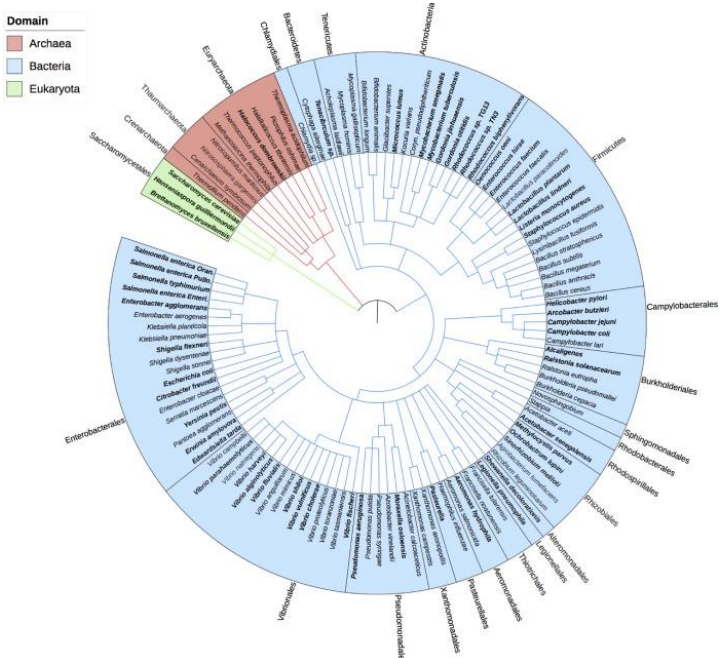
- Stochastic differences in intracellular pH
- ATP depletion
- Inhibition of DNA replication
- Toxin induction
- Protein aggregation
- Reduction of Ribosomes



Pu et al., 2019, Molecular Cell 73, 143–156
Goode et al. 2021; https://doi.org/10.1128/mBio.00909-21

Bacterial dormancy - VBNC

- Currently described for more than 100 different bacterial species, including 67 pathogenic bacteria
  - *Campylobacter spp., Escherichia coli, Francisella tularensis, Helicobacter pylori, Legionella pneumophila, Listeria monocytogenes, M. tuberculosis, Pseudomonas aeruginosa, Salmonella spp., Shigella spp.,Vibrio spp.*



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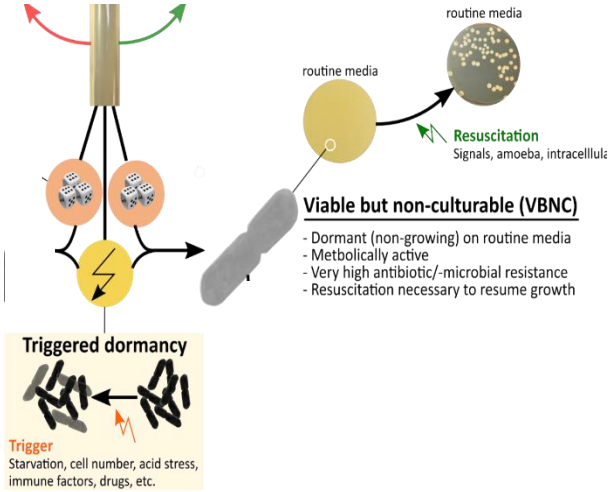
Bacterial dormancy - VBNC



- The VBNC state is a **universal survival strategy** for potentially the whole population
- The passive defense of VBNCs is reliant on cells entering a **dormant state**
- An external **signal / trigger is necessary** to leave the dormant state
- It's an **umbrella term** describing different physiological states

➔ There is **no** consensus paper clearly defining VBNC

➔ **No** consensus on how methods can be **evaluated and tested**

Persister vs VBNC

- Antibiotic persistence is **a universal survival strategy** where only a small subpopulation is highly tolerant
- The passive defense of persisters is reliant on cells entering a **dormant state**
- Cells by **replenishing** their energy level and **reactivating** cellular pathways
- It's an **umbrella term** describing different physiological states
- Definitions and guidelines exist

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- An external **signal / Trigger is necessary to leave** the dormant state
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- No Definitions and guidelines exist

Persister vs VBNC

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Research article

Viable but non-culturable and persistence describe the same bacterial stress state

Jun-Seob Kim, Nityananda Chowdhury, Ryota Yamasaki, Thomas K. Wood

First published: 19 February 2018 | https://doi.org/10.1111/1462-2920.14075 | Citations: 152

Opinion

'Viable but non-culturable cells' are dead

Sooyeon Song, Thomas K. Wood

First published: 15 March 2021 | https://doi.org/10.1111/1462-2920.15463 | Citations: 36

Correspondence

How dead is dead? Viable but non-culturable versus persister cells

Alexander K. T. Kirschner, Julia Vierheilig, Hans-Curt Flemming, Jost Wingender, Andreas H. Farnleitner

First published: 26 April 2021 | https://doi.org/10.1111/1758-2229.12949 | Citations: 6

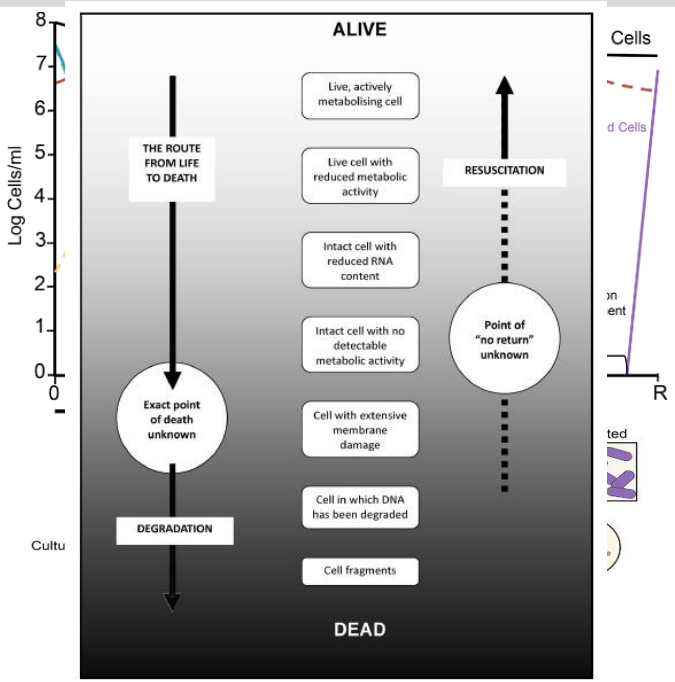
Correspondence

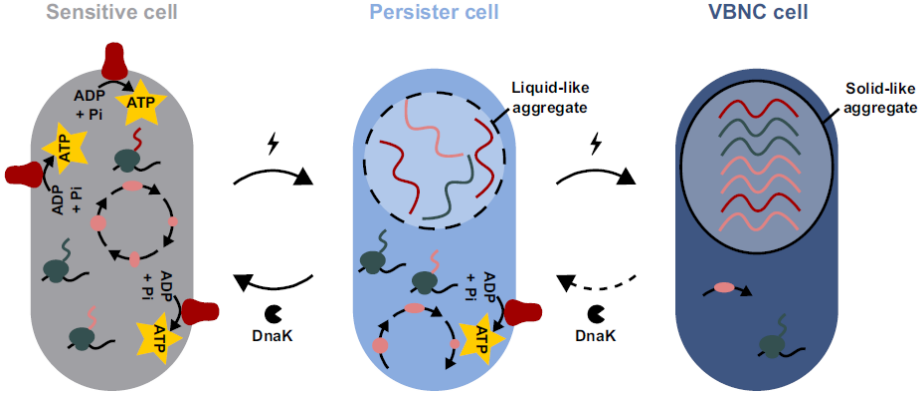
Waiting for Godot: response to 'How dead is dead? Viable but non-culturable versus persister cells'

Sooyeon Song, Thomas K. Wood

First published: 21 April 2021 | https://doi.org/10.1111/1758-2229.12951 | Citations: 2

Persister vs VBNC





- There seems to be a transition from persister to VBNC state

Ayrapetyan et al. (2018) doi: 10.1128/JB.00249-18.
Davey 2011; doi:10.1128/AEM.00744-11

Bollen et al. (2025) Nature Communications, https://doi.org/10.1038/s41467-025-56387-8.

Dormancy in the food sector

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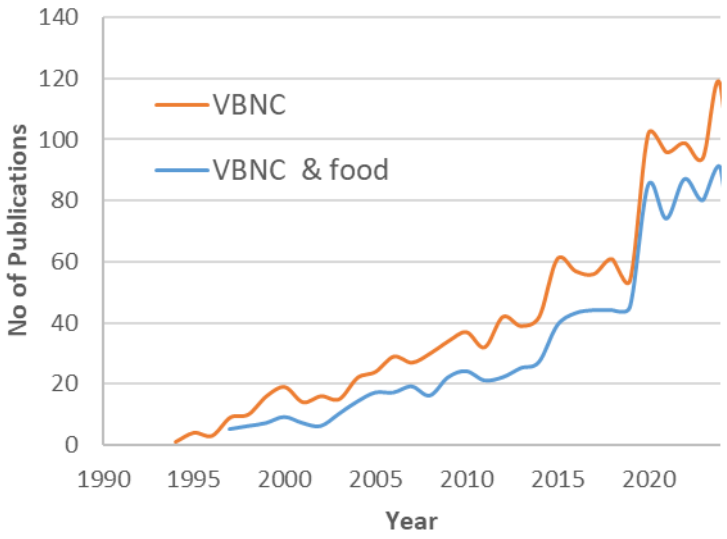


VBNC in the food sector

Temperature shifts

pH and Aw shifts

Use of disinfectants and cleaning agents

Constant entry of microorganisms

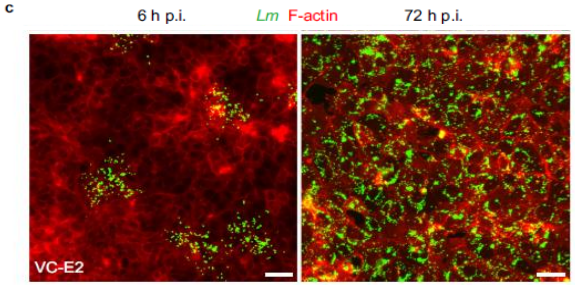
Nutrient rich environments

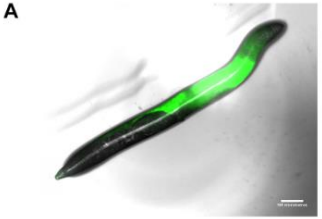
Nutrient depletion

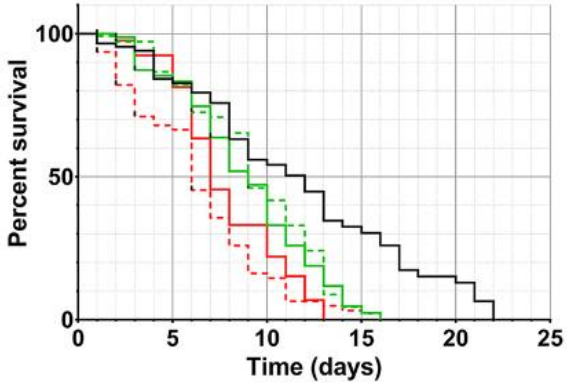
Predominantly growth-based methods for risk assessment

Challenges posed by VBNCs

- Retaining metabolic activity
  - Fermentation problems (e.g. juices, w
  - Toxin production
- Virulence
  - Infectivity of VBNC cells
  - Multiplication within host cells (or host) without regaining culturability
- Tolerance
  - Resistant to certain antibiotics
  - Increased tolerance against certain biocides
- Resuscitation
  - Reoccurrence through regrowth







Carvalho et al. 2024; Nature Communications; doi:10.1038/s41467-024-52633-7
Higmore et al. 2018; mBio; doi:10.1128/mBio.00540-18

Challenges posed by VBNCs

- Retaining metabolic activity
  - Fermentation problems (e.g. juices, wine or beer)
  - Toxin production
- Virulence
  - Infectivity of VBNC cells
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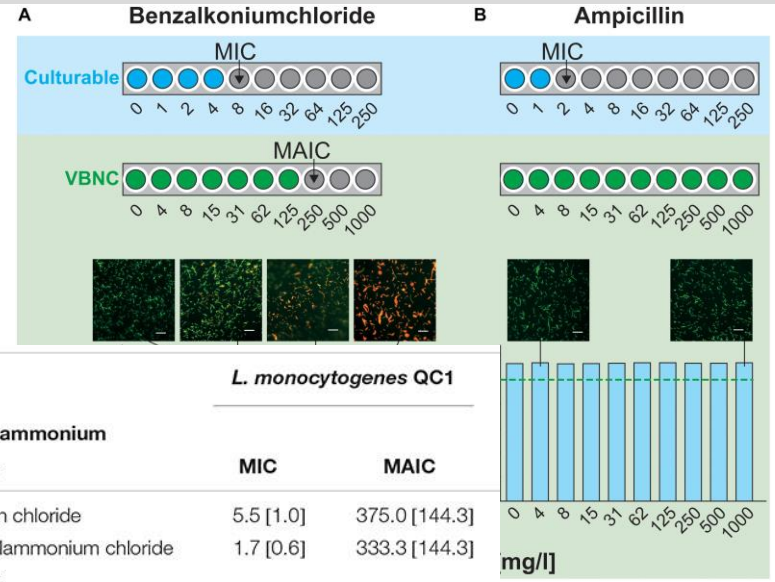

Quaternary ammonium compounds	MIC	MAIC
Benzalkonium chloride	5.5 [1.0]	375.0 [144.3]
Trioctylmethylammonium chloride	1.7 [0.6]	333.3 [144.3]
Preservatives		
Bronopol	62.0 [0]	666.7 [288.7]
Sodium azide	250.0 [0]	>1000 [0]

Mean MIC/MAIC values (mg/l) of QACs and preservatives after 24 h inc

Robben et al. 2019; DOI: 10.3389/fmicb.2019.01365

Challenges posed by VBNCs

- Retaining metabolic acti
  - Fermentation problems (e.g
  - Toxin production
- Virulence
  - Infectivity of VBNC cells
  - Multiplication within host cells (or host) without regaining culturability
- Tolerance
  - Resistant to certain antibiotics
  - Increased tolerance against certain biocides
- Resuscitation
  - Reoccurrence through regrowth

Table 1 | VBNC *Lm* revert back to a culturable state after passage in embryonated chicken eggs

Inoculum	Culturability before egg passage [a]	Culturability after egg passage [b]			
		Embryonated eggs	*p*-value [c]	Non-embryonated eggs	*p*-value [d]
Mineral water	0/3 (0%)	0/10 (0%)	>0.999	0/10 (0%)	>0.999
VBNC *Lm*	8/84 (9.52%)	24/24 (100%)	1.63×10^{-17}	0/18 (0%)	0.344
Vegetative *Lm*	3/3 (100%)	8/8 (100%)	>0.999	2/2 (100%)	>0.999

[a]Number of BHI wells with bacterial growth/Number of BHI wells inoculated.
[b]Number of eggs with bacterial growth/Number of eggs inoculated.
[c]Comparison of culturability before and after passage in embryonated eggs (two-sided Fisher's exact test).
[d]Comparison of culturability before and after passage in non-embryonated eggs (two-sided Fisher's exact test).

		Inoculation in embryonated eggs		Inoculation in non embryonated eggs
		Vitellus fluid[b]	Vitellus fluid and embryo[c]	
ATCC 19115	2	0/4	2/4	0/4
	6	0/4	2/4	1/4
LO 28	2	0/4	2/4	0/4
	6	0/4	2/4	0/4
Scott A	2	2/4	2/4	0/4
	6	4/4	4/4	0/4
CNL 895807	2	0/4	2/4	0/4
	6	0/4	2/4	0/4
Total		6/32	18/32	1/32
Negative control[d]	2	0/2	0/2	0/2
	6	0/2	0/2	0/2

Carvalho et al. 2024; Nature Communications; doi:10.1038/s41467-024-52633-7
Cappelier et al. 2006:DOI: 10.1051/vetres:2007017

What is the risk of VBNCs

- Retaining metabolic activity
  - Fermentation problems (e.g. juices, wine or i
  - Toxin production
- Virulence
  - Infectivity of VBNC cells
  - Multiplication within host cells (or host) without regaining culturability
- Tolerance
  - Resistant to certain antibiotics
  - Increased tolerance against certain biocides
- Resuscitation
  - Reoccurrence through regrowth

Problematic umbrella terms

- How do bacterial cells regulate the VBNC state?
  - Dormancy / Tolerance / Metabolic activity / Virulence
- How comparable are results between species?
- Is there only one VBNC state?
  - Dependent on the induction conditions?
  - Different subpopulations?

Effect of induction conditions

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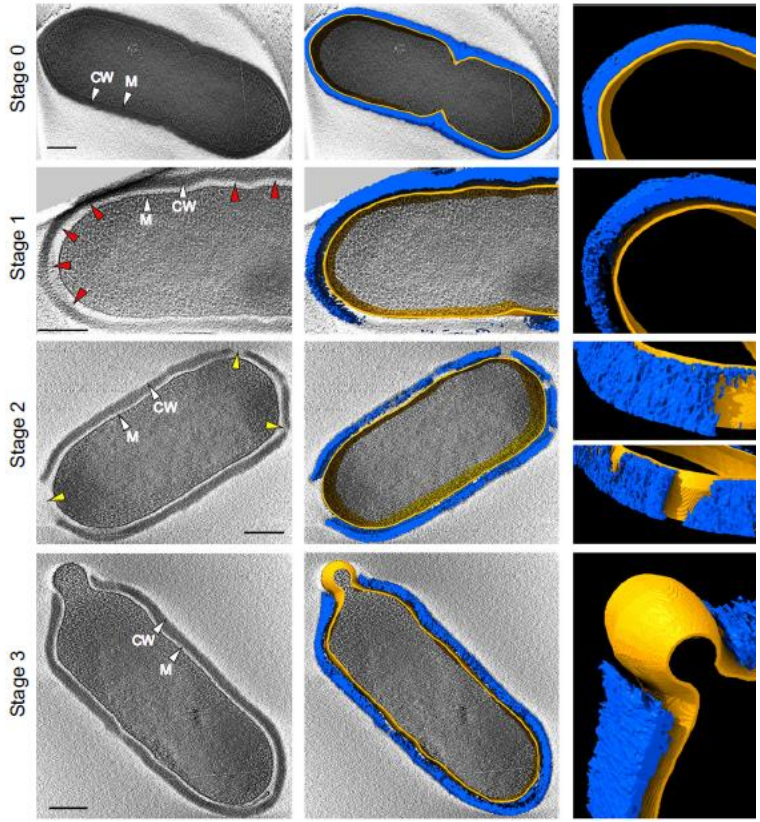
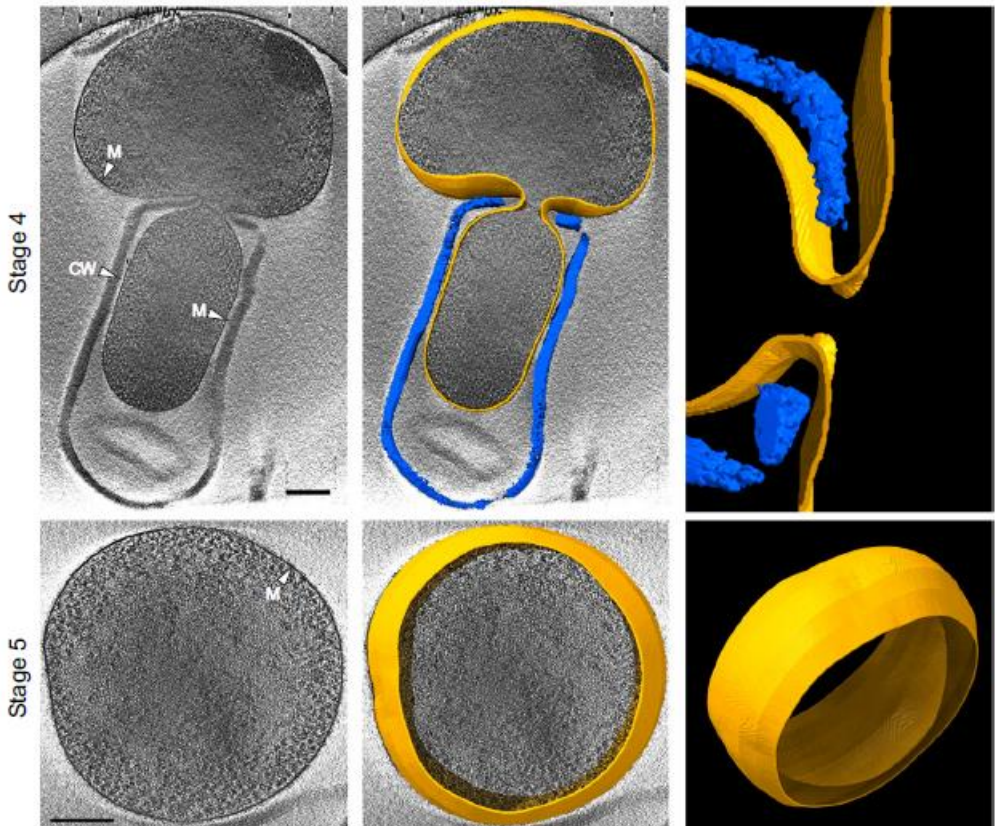
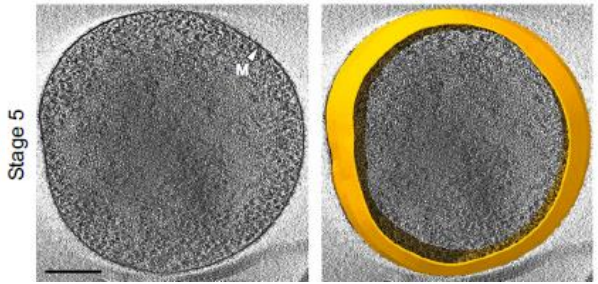
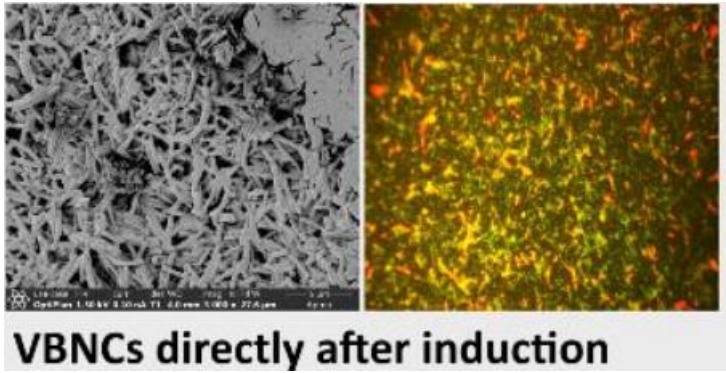


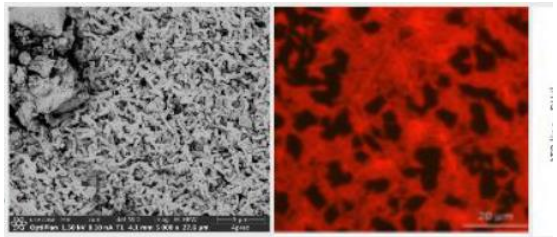


Effect of induction conditions

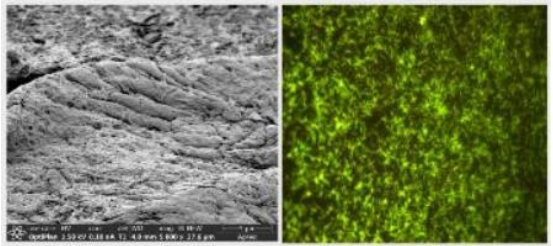




VBNCs directly after induction



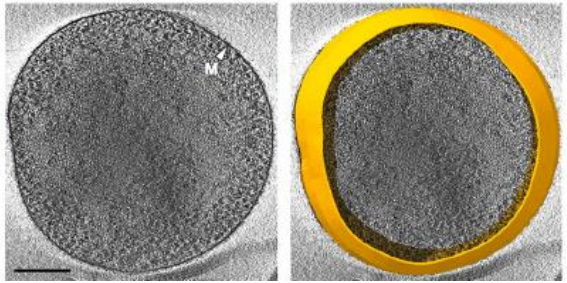
Heat inactivated VBNCs stored in BHI



VBNCs stored in BHI

Carvalho et al. 2024; Nature Communications; doi:10.1038/s41467-024-52633-7
Mikuni-Mester unpublished

Effect of induction conditions



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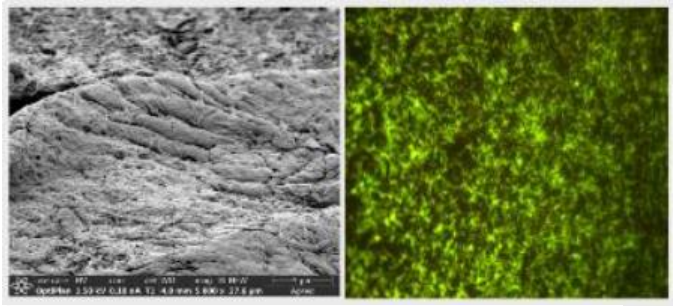

Quaternary ammonium compounds	*L. monocytogenes* QC1 MIC	MAIC
Benzalkonium chloride	5.5 [1.0]	375.0 [144.3]
Trioctylmethylammonium chloride	1.7 [0.6]	333.3 [144.3]
Preservatives		
Bronopol	62.0 [0]	666.7 [288.7]
Sodium azide	250.0 [0]	>1000 [0]

Mean MIC/MAIC values (mg/l) of QACs and preservatives after 24 h inc

Carvalho et al. 2024; Nature Communications; doi:10.1038/s41467-024-52633-7
Mikuni-Mester unpublished
Robben et al. 2019; DOI: 10.3389/fmicb.2019.01365

Open questions and few answers

- How do bacterial cells regulate the VBNC state?
- How comparable are results between species?
- Is there only one VBNC state?

- Lacking validation of analytical tools to detect and quantify VBNCs
  - Viability PCR is most widespread but not without pitfalls

Propidium Monoazide is Unreliable for Quantitative Live–Dead Molecular Assays

Simerdeep Kaur, Laura Bran, Grigorii Rudakov, Jiangshan Wang, and Mohit S. Verma*

Open questions and few answers

- How do bacterial cells regulate the VBNC state?
- How comparable are results between species?
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- Lacking validation of analytical tools to detect and quantify VBNCs
  - Viability PCR is mots widespread but not without pitfalls
- How does the Food production environment influence VBNC?
  - Which process steps or conditions induce the VBNC state
- How can we effectively reduce persistent cells in the VBNC state?

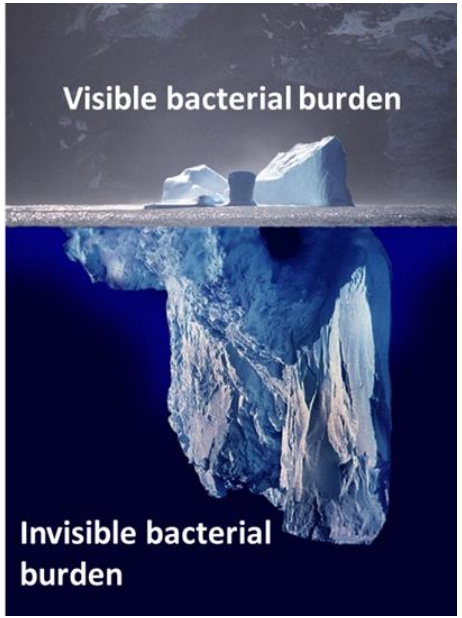
Summary

- Pathogenic bacteria can enter dormancy states either spontaneously or as a response to stress in which there are viable but non-culturable under routine conditions
- Cells in the VBNC state can **be highly resistant** against certain antibiotics, antimicrobials or physical stress which allow them **to persist in food production** environments
- Cells in the VBNC state can be detected
- However…..

Control of VBNC bacteria

- Effective measures against VBNCs are currently not established
  - Tolerance differs between species and induction
  - Research is focused on studying tolerance/resistance and not trying to identify effective measures
  - Problematic lack of standards
  - Research is often more Problem instead of Solution orientated

Bacteria in the VBNC state are **NOT** an emerging or new threat



Challenges posed by VBNCs

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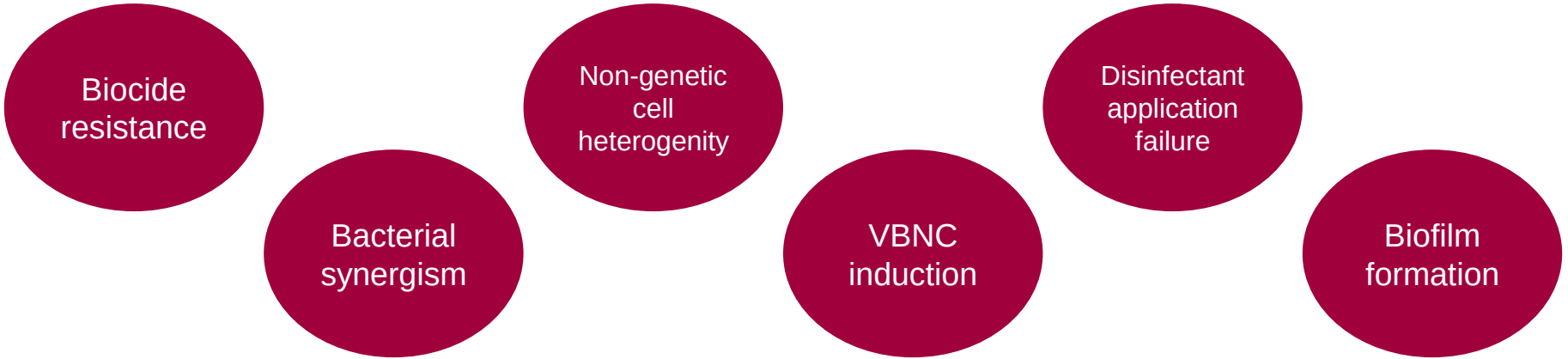
Persistence of bacteria in food production Environments



Summary

- Pathogenic bacteria can enter dormancy states in which they are highly resistant
- In the VBNC state cells are not-detectable to growth-based methods allowing them **to persist in food production** environments
- By **combining the appropriate and validated methods** the resistance of cells in the VBNC state can be analyzed and effective interventions identified
- We need to identify and validate **meaningful detection methods** that are economically feasible complementing existing technologies

Take Home Message

The realization of widespread bacterial dormancy (VBNC, Persister etc.) together with the development of detection methods and effective intervention measures will help us to reduce **already existing** problems