MICROBIAL STRESS,

GROWTH, AND PRODUCE TESTING:

HOW BACTERIA ADAPT IN OUR FOOD SAMPLES & SYSTEMS

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COMPANY OVERVIEW

Eurofins is the **global leader in biological testing** with an unrivaled reputation for unbiased analysis

200,000 reliable analytical methods for characterizing the safety, identity, purity, composition, authenticity, and origin of products

Our **diverse laboratories** navigate seamlessly through a dynamic and ever-changing global marketplace

50K+ EMPLOYEES

800+ LABORATORIES

50 COUNTRIES

400M+ TESTS ANNUALLY

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AGENDA

- Microbial Stress Responses
- VBNC
- Impacts on testing
- Microbiomes & microbial dynamics

ORIGINS

Bacteria have been on Earth for at least 3.5 billion years

Believed to be the first life forms

Continue to dominate species (78% are from bacteria)

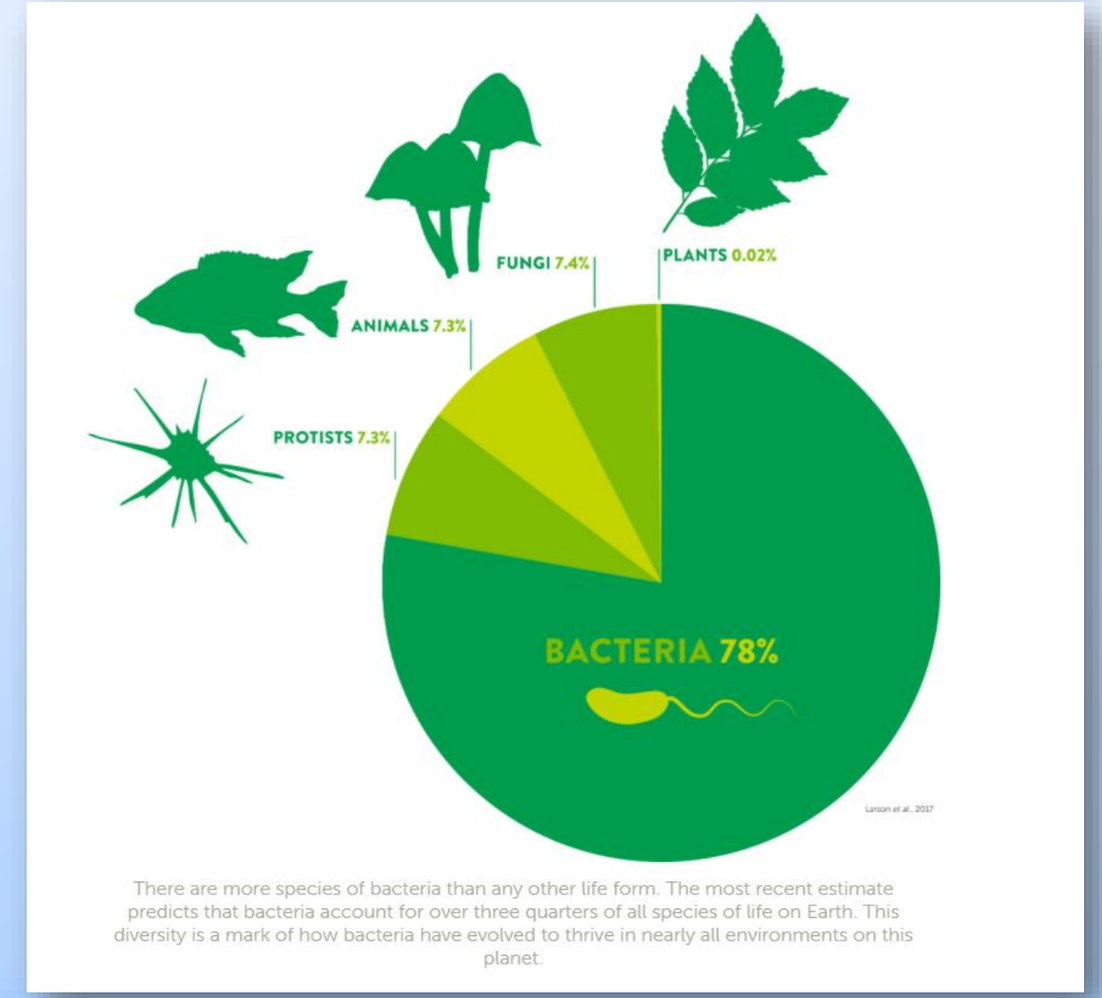


Image source: http://www.oum.ox.ac.uk/bacterialworld/

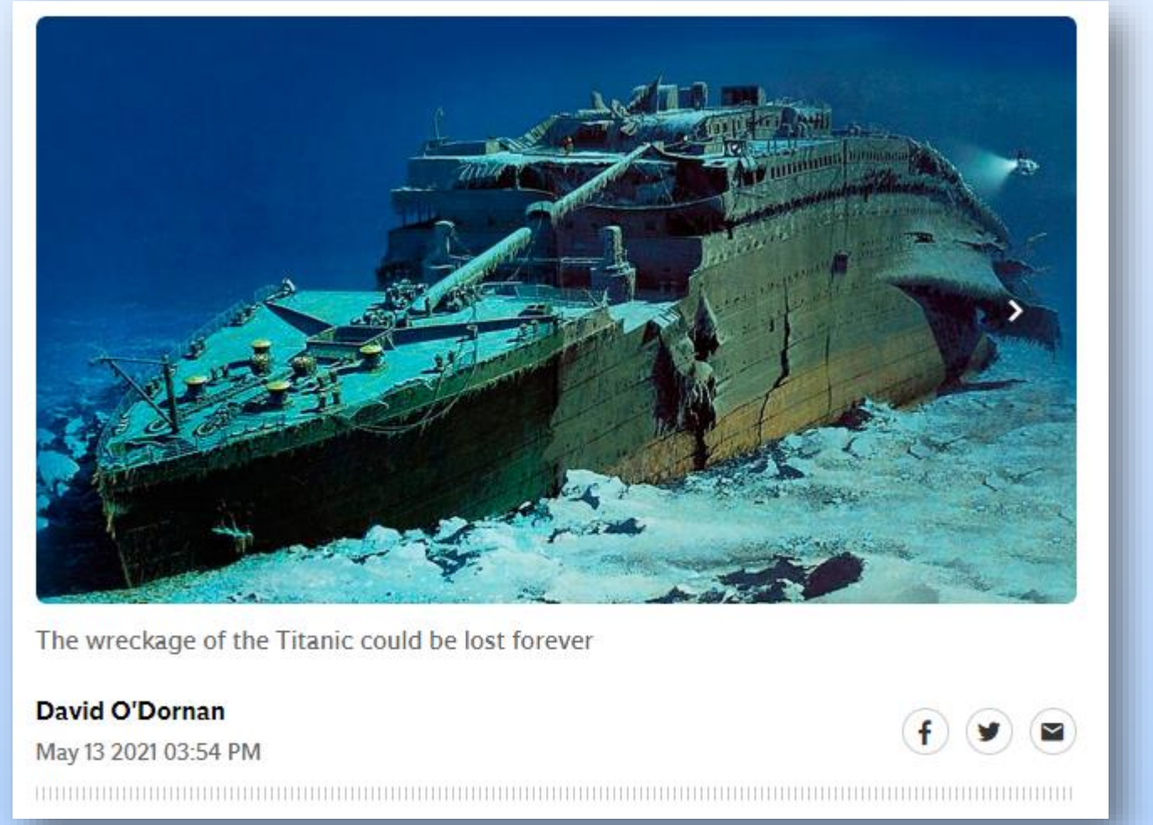
ORIGINS

- **Survive in all locations on the planet**
  - Antarctica
  - Hot springs/geysers
  - Anaerobic locations

- **Bacteria are highly responsive to environmental conditions**
  - Rapidly monitoring conditions
  - Experts at survival
  - Developed numerous mechanisms to protect themselves

TITANIC EATEN BY MICROORGANISMS

- Previously thought that the depth (pressure, cold, lack of oxygen, etc.) would preserve the ship (sunk 110yrs ago)
- Metal-eating bacteria consume 100kg of iron/day
- Estimated to fully consume the ship by 2030



The wreckage of the Titanic could be lost forever

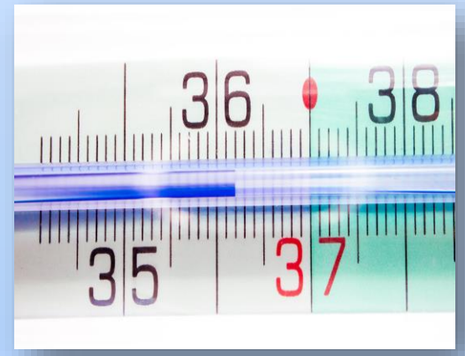
David O'Dornan

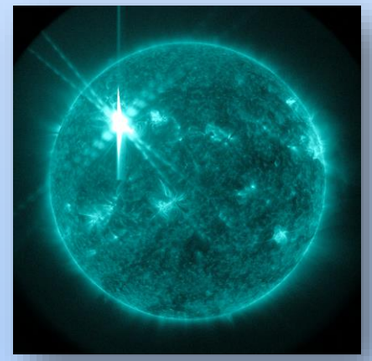
May 13 2021 03:54 PM

Source: https://www.belfasttelegraph.co.uk/news/northern-ireland/time-is-running-outto-savetitanicwreckage-warn-experts-40423575.html

BACTERIAL ORIGINS

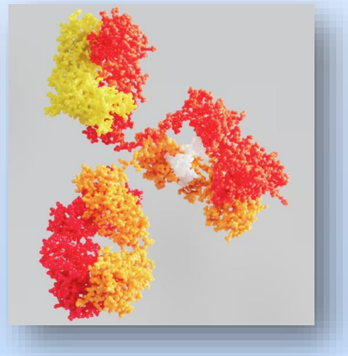
"What doesn't kill you makes you stronger"






SELECTING FOR SURVIVORS

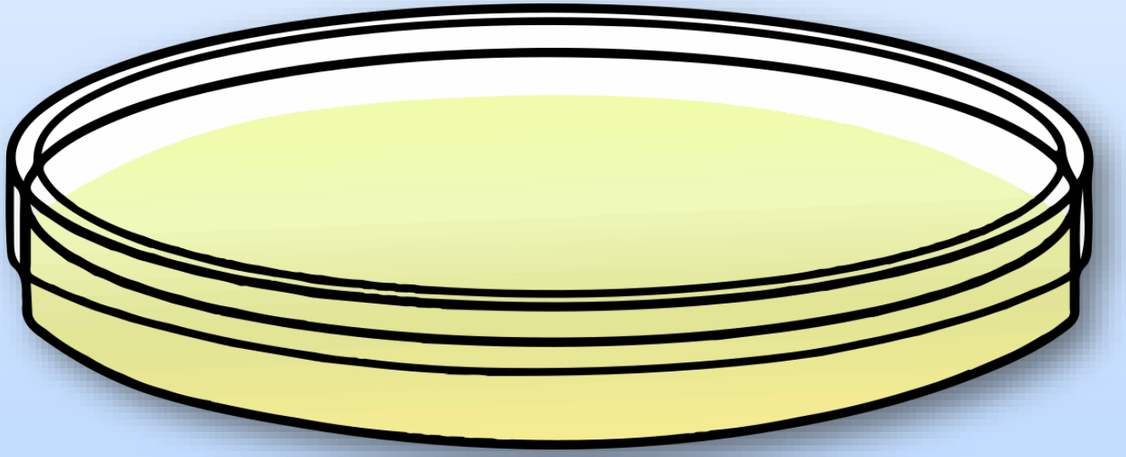
Microorganisms have developed many mechanisms to survive different conditions



- Many response pathways to repair damaged DNA and upregulate defensive modifications
- Heat shock proteins can help with damaged proteins
- Modifications to proteins and lipids
  - Thermophiles: increased ionic bonds & hydrophobic components of proteins to maintain structure
  - Halophiles: compatible solutes to balance intracellular osmotic pressure to extracellular osmotic pressure
  - Psychrophilic: more pockets of space to minimize ice formation & maintain fluidity

VIABLE BUT NONCULTURABLE (VBNC)

- **Survival advantage to harsh conditions**
  - Nutrient starvation
  - Extreme temperatures
  - Changes in salt, pH, osmotic stress
  - Oxygen availability
  - Intense light exposure
  - Pressure/heat exposure
  - Chlorine exposure
- **Cells enter a dormant state**
  - Very limited metabolic activity
  - Resistant to some antibiotics
  - Cannot be cultured using traditional culturing techniques
  - There is evidence that cells can be resuscitated to full metabolic activity
  - Unknown impact within the food production systems



RELEVANCE TO THE FOOD INDUSTRY

- **Target organisms in food industry that have been shown to have robust stress & VBNC states:**
  - *Listeria species*
  - *Listeria monocytogenes*
  - *Salmonella*
  - *E.coli*
  - Pathogenic *E.coli*
  - *Vibrio*
  - *Campylobacter*
  - *Pseudomonas spp.*
  - *Shigella*

- **Main point:** *Bacteria are adaptable & have evolved to SURVIVE. Food systems can be challenging environments for microbes.*

STRESSED & INJURED CELLS

- **Cells that are stressed or injured may have slower growth rates**
  - Lag time & optimal growth rate

- **Microbiological methods are designed for target organisms**
  - Medias with selective agents
  - Temperatures
  - Inhibitors/antibiotics
  - Certain biologic conditions

- **Methods can be designed to help resuscitate injured/dormant cells**
  - Non-selective media, then selective
  - Lower temperature enrichments (e.g. 25C for a time period, then a selective temperature to follow)
  - Additives/Supplements

EXAMPLES OF GROWTH RATE CHANGES

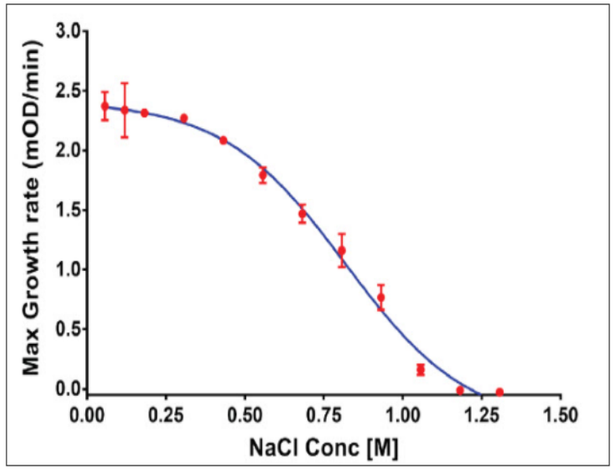


Figure 4. Growth rate of *E. coli* with various concentrations of bacterial media. LB and 2XYT media was diluted with water and inoculated with E. coli and growth monitored for kinetically. Maximum growth rate for each well was determined and plotted as a function of growth media concentration. Data represents the mean and standard deviation of eight determinations.

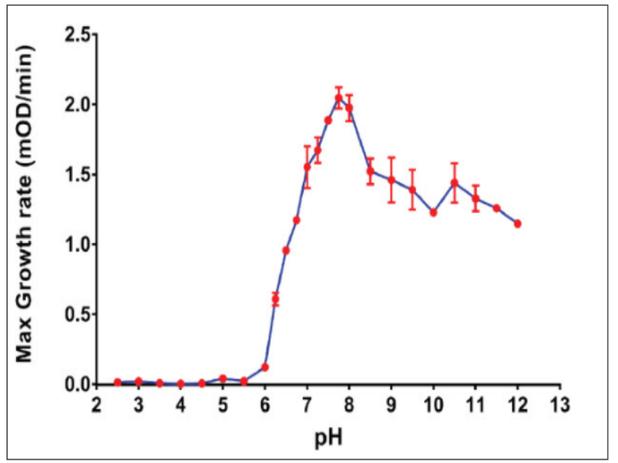


Figure 5. Effect of media pH on bacterial growth. 100 µL of dilute *E. coli* cultures in 2XYT media were added to wells of a microplate with 50 µL of universal buffer at various pH levels. Growth was monitored kinetically for 12 hours and the maximum growth rate plotted as a function of pH. Data represents the mean and standard deviation of eight determinations.

Source:

BioTek by Agilent

https://www.biotek.com/resources/application-notes/m-onitoring-bacterial-growth-under-different-environmental-conditions/

Accessed 11/29/2021

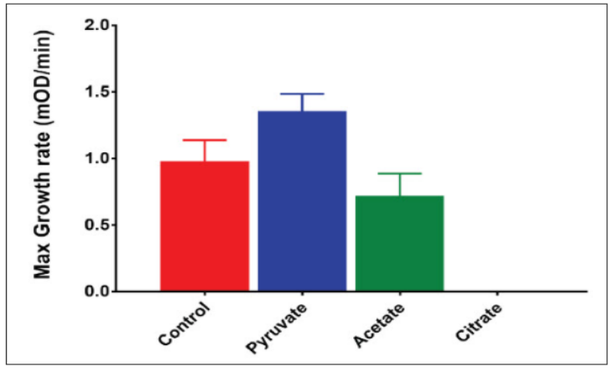


Figure 7. Comparison of bacterial growth rate in the presence of small carbon molecules. 2XYT media, supplemented with 50 µM of the indicated molecule and 50 mM Hepes (pH 7.5), was inoculated with *E. coli* and the growth monitored kinetically. Bar graph represents the mean and standard deviation of eight determinations.

SALMONELLA GROWTH VARIABILITY

- **Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior.** 2011. Food Microbiology. 28: 828-837.

- Studied 60 isolates of *Salmonella enterica* at varying pH and Aw (NaCl concentrations) to observe changes to the maximum growth rate
  - Study to evaluate changes between serovars
  - Study to understand changes within a serovar
  - Provide growth rate observations for modeling & method development

- Findings of strain variability (serovars):
  - pH 7.0 -0.5% NaCl coefficient of variation was **6.1%**
  - pH 4.3-0.5% NaCl coefficient of variation was **11.8%**
  - pH 7.0-6.0% NaCl coefficient of variation was **23.5%**

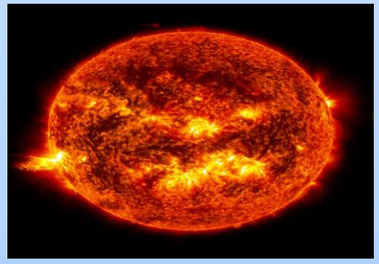
Image source: https://www.fda.gov/food/foodborne-pathogens/salmonella-salmonellosis

RELEVANCE TO THE FOOD INDUSTRY

- **Common processes & conditions may induce the VBNC state, or stress responses for pertinent bacteria**
  - Pasteurization
  - Chlorine washes/dips
  - Fast temperature shifts (high heat/low temperature)
  - Increased salts/preservatives
  - Increased Pressure

- **Unique Produce industry considerations**
  - Extreme weather events & temperature shifts
  - Limited nutrient availability on crop surfaces/soils
  - UV exposure
  - Desiccation
  - Antimicrobial rinses

PRODUCE ENVIRONMENTAL CHALLENGES



https://extension.usu.edu/yardandgarden/research/gardening-in-clay-soils
https://www.producebluebook.com/2020/08/21/heat-and-fires-disrupt-salinas-crops/#

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IMPACT FOR FOOD SAFETY

- Microorganism state may vary day-to-day, within the process, by supplier, by weather….etc.

- Consider your product & process for:
  - Risk assessment on what organisms may be best suited for survival in your product
  - Determine if there are points in time when that risk may change

- Be aware of trade-offs in your testing plan:
  - Speed vs. sensitivity
  - Budgeting for stress & resuscitation
  - Research organism behavior in your system

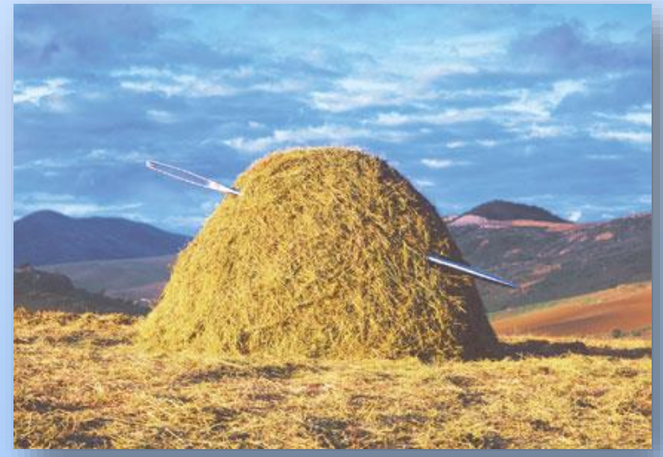
TESTING CONSIDERATIONS

We are looking for “low levels” of contamination. 1 CFU in 25–375g.

Most current rapid methods require a “growth step” to increase numbers of cells to 10,000 CFU.

What impacts growth?

- Injury
- Competition
- Limits of biology
- Stress
- Nutrients available
- TIME!!!



ENRICHMENT CONSIDERATIONS

https://www.amazon.com/Hallmark-Keepsake-Christmas-Ornament-Family

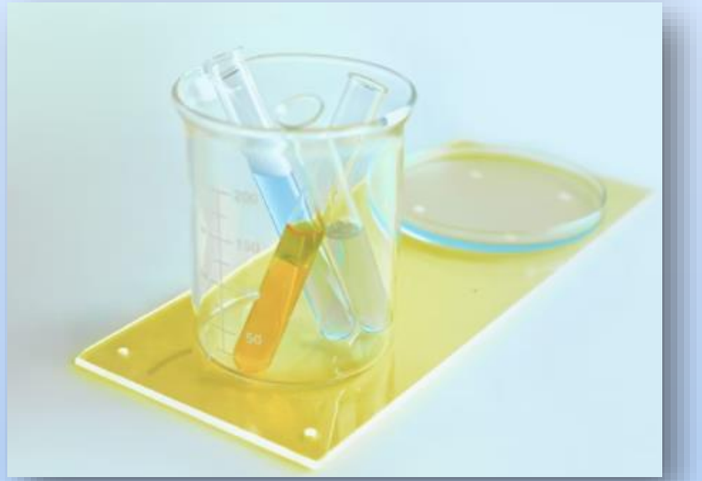
FASTER PLAYERS WILL EAT UP MORE FOOD & WIN THE GAME

IF ONE PLAYER HAS MORE TIME, THEY WILL BE ABLE TO GET MORE FOOD

IF ONE PLAYER IS BROKEN/INJURED, THEY WILL HAVE A HARDER TIME COMPETING

VISUALIZING AN ENRICHMENT

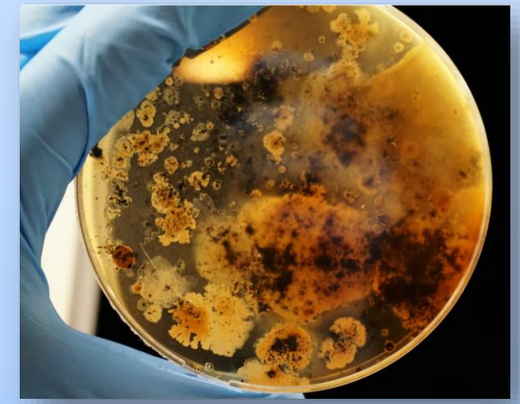
- Common detection methods are designed to identify one organism, but an enrichment can be quite complex



- Enrichment medias are critical pieces of a method
  - Determines what grows
  - Inhibits what you don’t want
  - Resuscitates organisms
  - Minimizes inhibitors in product/samples
- Enrichment protocols are then paired with detection technologies
  - PCR, immunoassays, LAMP, biosensors, etc.

VISUALIZING AN ENRICHMENT

- New technologies in genomic testing offer a glimpse into the unknown
  - Genetic information on everything present
  - Based on DNA & RNA approaches
  - Different methods can target pieces of genome, or complete assembly of genome
  - No culture bias (growing what we know is there)
  - Can be completed before enrichment, or after an enrichment

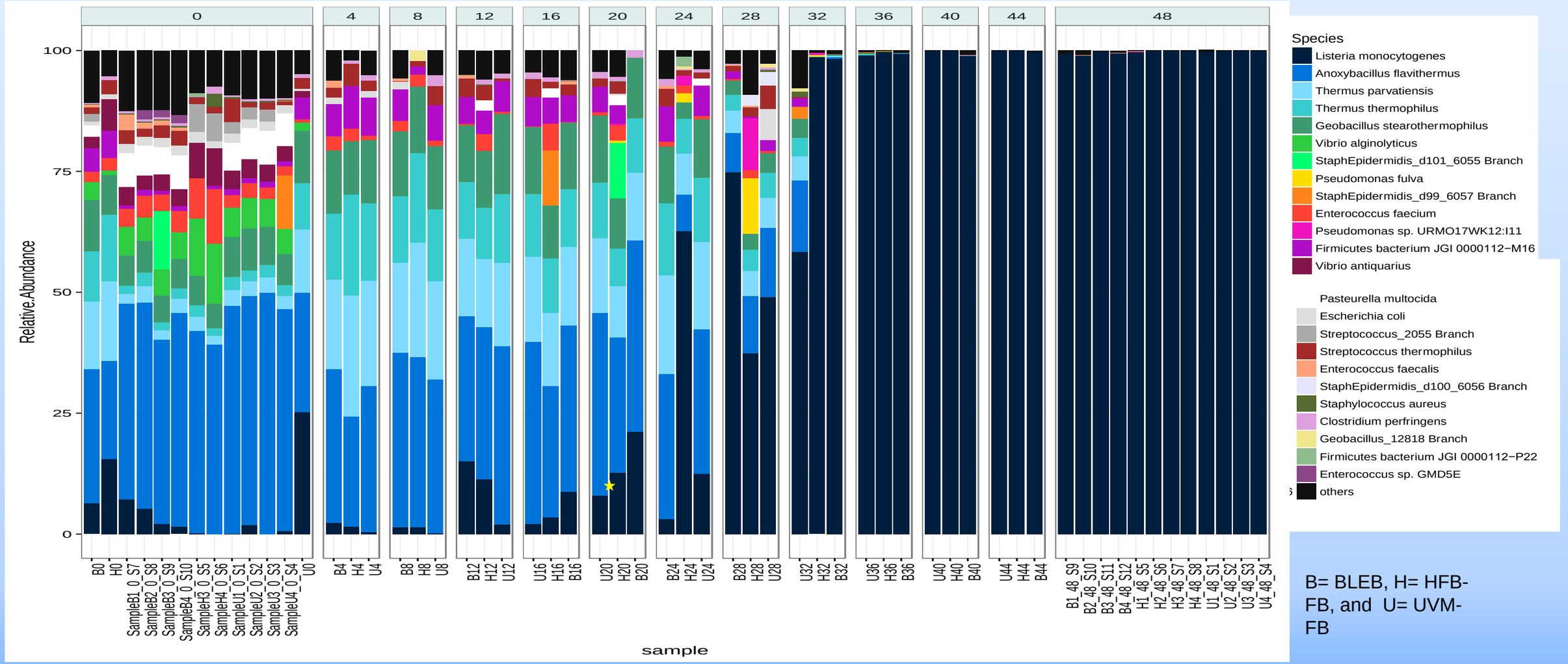


WGS OPTIONS

- Different technologies are available today:
  - Shotgun sequencing
  - Long read sequencing
  - 16S & 18S/ITS
  - MLST
  - Sanger sequencing
  - etc.

- Rapidly evolving industry
  - Sensitivity too low for detection methods (at this point) in common food sample sizes
  - Cost too high for regular detection methods
  - Strain sequencing proving useful for investigations & surveillance
  - Microbiomes proving useful in investigations & spoilage studies

ENRICHMENT MICROBIOME PROFILES (ICE CREAM)



B= BLEB, H= HFB-FB, and U= UVM-FB

Ottensen et al., BMC Micro, 2016; figure courtesy of N. Hassan, CosmosID

LETTUCE ENRICHMENT STUDY

- **Lettuce microflora can be often be 10^3 – 10^7 CFU/g**
- **Detection methods tell us an answer, but not the whole picture**
  - End result
  - No visibility to what is happening in the broth
- **Validations are built off of lab conditions, not real-world conditions**
  - Designed to try to capture some microbial stress, but limited to practical lab protocols
  - Validations don’t specify what strains to study

LETTUCE ENRICHMENT STUDY – AN EXAMPLE

- Designed to evaluate the impact of cold stress on *E.coli* O26 and background microflora
- An opportunity to utilize WGS and microbiome analysis to “see” microbial growth dynamics
- *E.coli* O26 inoculum was unstressed & cold stressed
- Romaine lettuce was inoculated with *E.coli* O26 (STEC) at high, medium, and low levels

INOCULUM & SAMPLE PREPARATION

Overnight culture of *E.coli* O26 (10^8 CFU/ml)

- Three inoculation levels:
  - Low = 1 CFU per sample
  - Medium = 10 CFU per sample
  - High = 92 CFU per sample
- Unstressed vs. Stressed conditions
  - On product cold stress (4C, 48hrs)
  - Direct inoculation, then enrichment

DETECTION

Enrichment time (hr)	Inoculum level (CFU/375g)		Real-time PCR: BAX STEC screen	Real-time PCR: BACGene STEC screen
4hr	10	Stressed	Negative	Negative
			Negative	Negative
			Negative	Negative
	1		Negative	Negative
			Negative	Negative
			Negative	Negative
	92	Unstressed	Positive	Negative
			Positive	Negative
			Positive	Positive
	10		Negative	Negative
			Negative	Negative
			Negative	Negative
	1		Negative	Negative
			Negative	Negative
			Negative	Negative
6hr	10	Stressed	Positive	Negative
			Positive	Positive
			Positive	Positive
	1		Positive	Negative
			Positive	Negative
			Positive	Negative
	92	Unstressed	Positive	Positive
			Positive	Positive
			Positive	Positive
	10		Positive	Positive
			Positive	Positive
			Positive	Positive
	1		Positive	Positive
			Positive	Positive
			Negative	Negative
			Positive	Negative
8hr	10	Stressed	Positive	Positive
			Positive	Positive
			Positive	Positive
	1		Positive	Positive
			Positive	Positive
			Positive	Positive
	92	Unstressed	Positive	Positive
			Positive	Positive
			Positive	Positive
	10		Positive	Positive
			Positive	Positive
			Positive	Positive
	1		Positive	Positive
			Positive	Positive
			Negative	Negative
			Positive	Positive

Enrichment time (hr)	Inoculum level (CFU/375g)		Real-time PCR: BAX STEC screen	Real-time PCR: BACGene STEC screen
10hr	10	Stressed	Positive	Positive
			Positive	Positive
			Positive	Positive
	1		Positive	Positive
			Positive	Positive
			Positive	Positive
	92	Unstressed	Positive	Positive
			Positive	Positive
			Positive	Positive
	10		Positive	Positive
			Positive	Positive
			Positive	Positive
	1		Negative	Negative
			Positive	Positive
			Positive	Positive
16hr	10	Stressed	Positive	Positive
			Positive	Positive
			Positive	Positive
	1		Positive	Positive
			Positive	Positive
			Positive	Positive
	92	Unstressed	Positive	Positive
			Positive	Positive
			Positive	Positive
	10		Positive	Positive
			Positive	Positive
			Positive	Positive
	1		Negative	Negative
			Positive	Positive

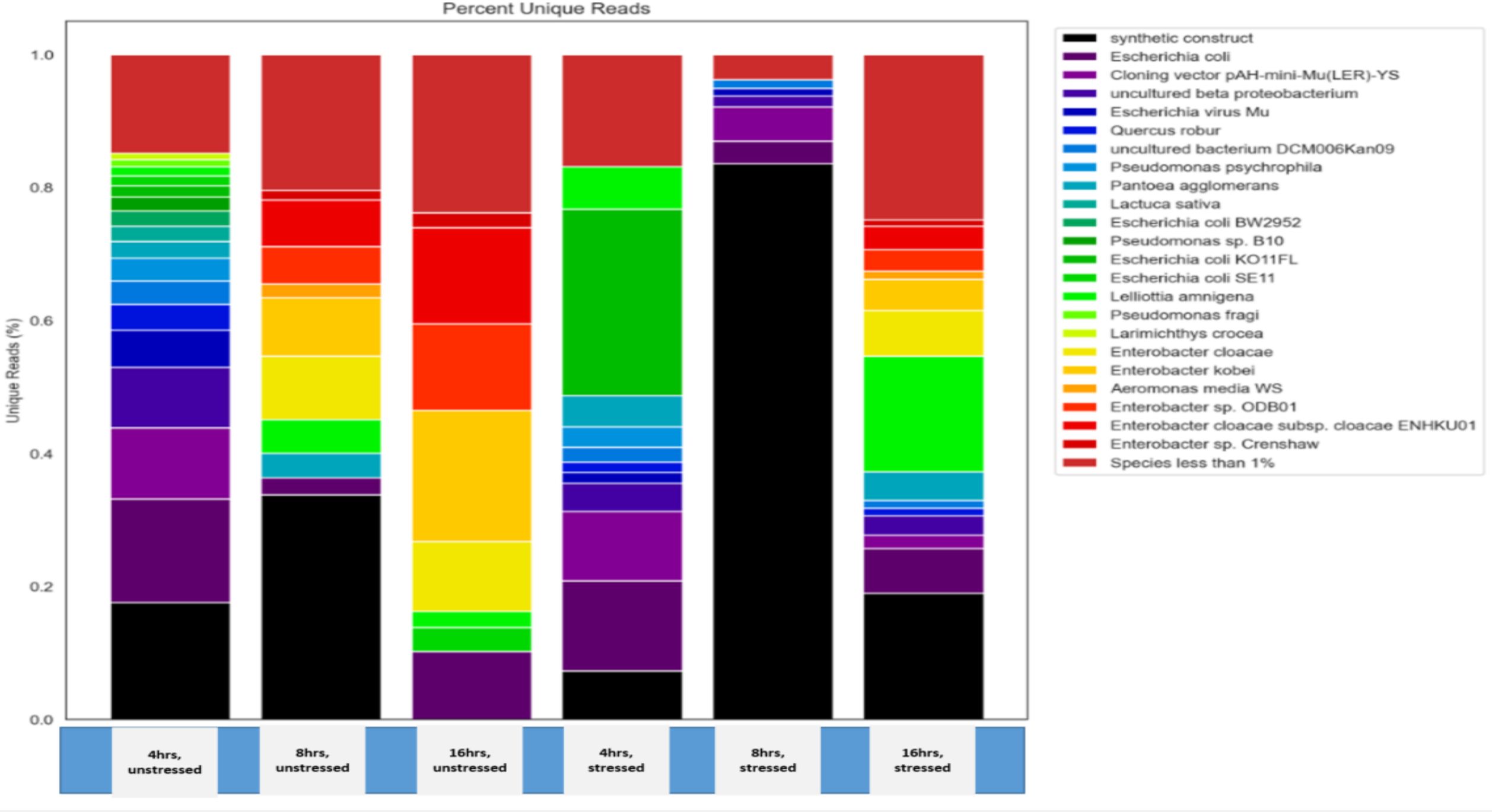


Figure courtesy of EzBiome

MICROBIOME OBSERVATIONS

- **Distinct population differences (rel. abundance & composition) was seen before the stressed & unstressed conditions**
- **A lower amount of DNA was recovered from the stressed & unstressed conditions**
  - Unused reagents seen in stressed conditions, indicating lower amount of DNA to sequence
  - Cold stress impacts native microflora as well as target organism
- **Diversity was highest at the early stage of enrichment (4hrs) & quickly began to change**
  - *Pseudomonas* counts decrease significantly from 4-8hrs of enrichments
  - Overall *E.coli* populations (including O26) grow to substantial proportions of the total population between 8-16hrs

SUMMARY

- **Microbial stress & injury can have major implications for food samples sent for testing**
  - Real-world conditions may vary day to day, sample to sample
  - Balance speed with understanding bacterial stress responses and potential injury
- **Validations are complicated**
  - Analyze what strains were included in the study, and what stress they may have been under for validation
  - Consider your process and product while optimizing enrichment conditions
- **It’s not just the target organism you need to think about**
  - New technologies open a world of visualizing communities – WGS sequencing
  - WGS showed us a difference in the diversity & quantity

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THANK YOU

LITERATURE SOURCES

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