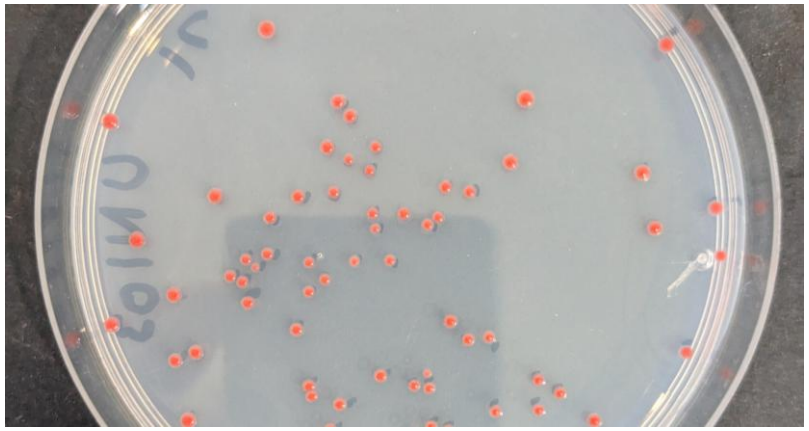
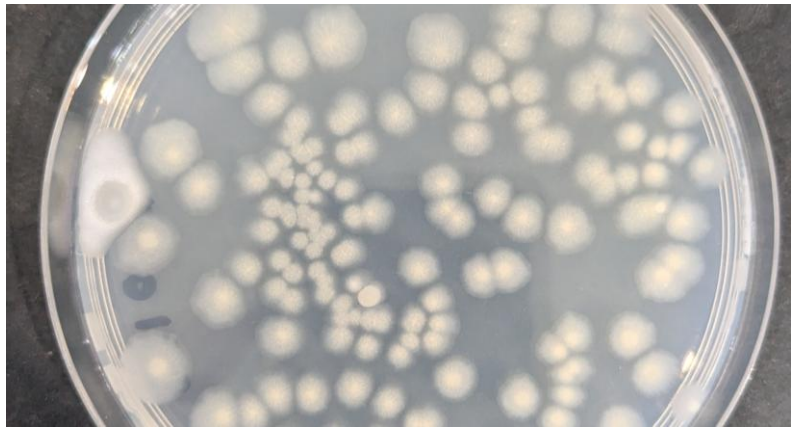
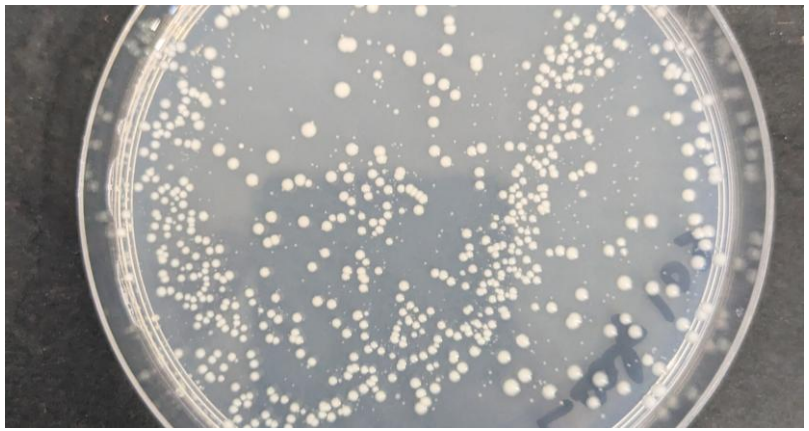
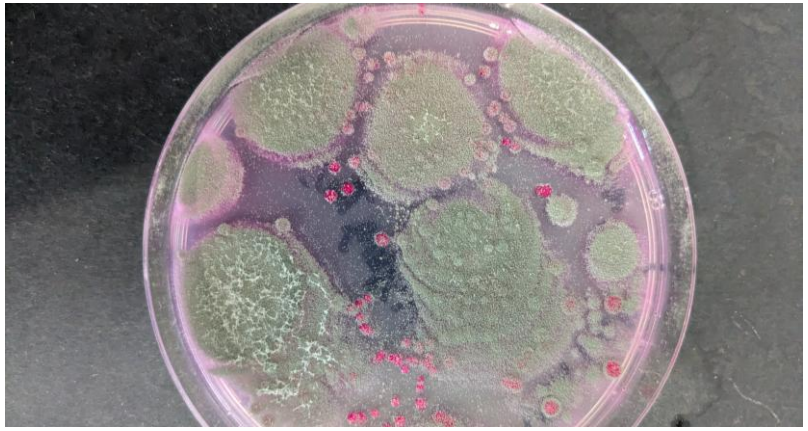
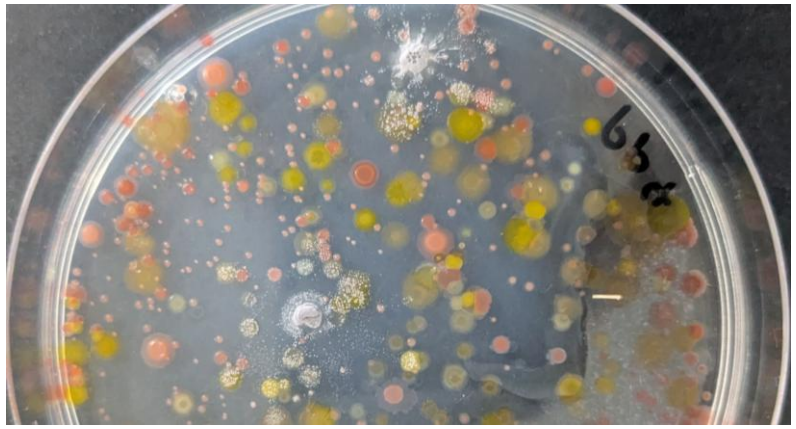
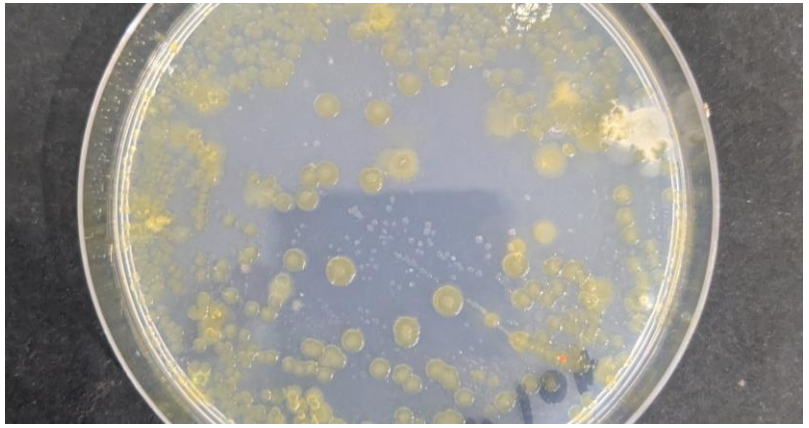
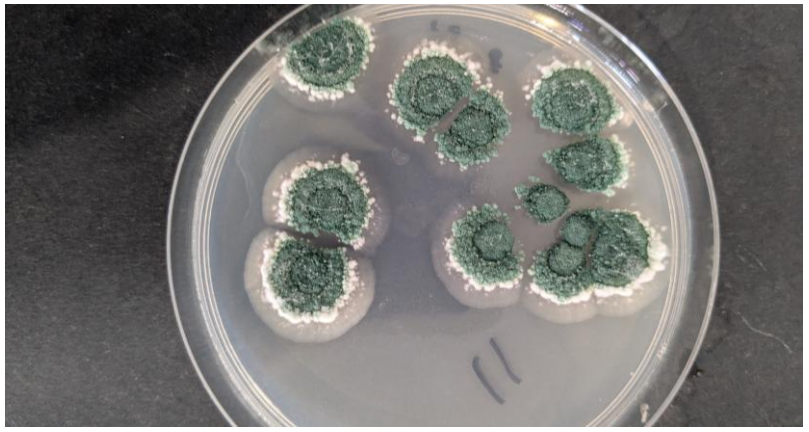








Confirming what is expected in live microbial products.

Rohit Krishna, Bachelor and Masters of Ag science, University of Queensland Gatton.

Dr. Sundar Kalaipandian, Research Fellow, University of Queensland Gatton

Dr. Benjamin Evert, Research and Development Scientist, Metagen Australia

Metagen Statement of Conflict of Interest

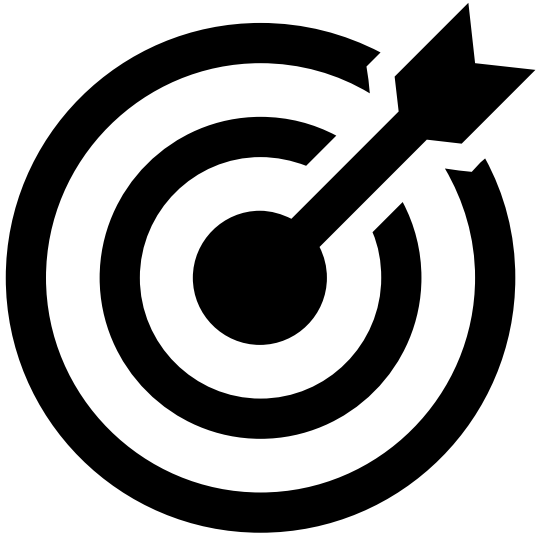
- This was a UQ masters project done at the Metagen laboratory. Metagen staff did not do the testing.
- Product and client names were blinded.
- Any collected products, isolates and DNA sequencing data have been disposed of.
- All parties involved in the project signed an NDA to protect the privacy of any products that were submitted or any client details that submitted samples.

metagen AUS

Project Aims

- Products were sent in.
- We were told what microbial species and concentration to expect in them.
- We tested them to see if that's what we found.

The project aimed to see how often the microbial species and concentration expected to be in live microbial products was found.



What exactly are live microbial products?

3. Definitions

A plant biostimulant is a substance(s), microorganism(s), or mixtures thereof, that, when applied to seeds, fertilizer, plants, the rhizosphere, soil or other growth media, act to support a plant's natural nutrition processes independently of the biostimulant's nutrient content.

The plant biostimulant thereby improves one or more of:

- nutrient availability, uptake, or use efficiency,
- tolerance to abiotic stress, and
- consequent growth, development, plant vigour, crop quality or yield.

Plant biostimulants can be placed into five compositional categories:

A. Microbial based biostimulants

1. Live microbial products
2. Complex products based on non-living microorganisms and their metabolites

- Fert Australia code of practice breaks plant and soil bio stimulants into 5 categories A-E.
- The types of products we tested are listed in the code of practice under A . 1. Live microbial products.
- They can have defined species of microbes and concentrations
- They can be undefined and contain 100s-1000s of different species of microbes at unknown concentrations.

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How exactly are these products labelled?

Usually, the name of the microbial species followed by colony forming units (CFU) per ml/g.

Bacteria X:
2.4 x 10^9 CFU/ml

Microbial biostimulants.
State the genus and species of each active ingredient that is a microorganism as well as the following information:
(i) for each microorganism that is a culturable cell, the number of colony forming units per gram or millilitre (CFU/g CFU/ml) of that microorganism, and
(ii) for each microorganism that is not a culturable cell, another descriptor of the concentration of that microorganism, or metabolite, on a per gram or millilitre basis, e.g. cells, spores or propagules per gram or millilitre, quantitative or digital PCR.

Non target species should be less than 1% of microorganisms in the product. The label should state "Other species < 1%".

This type of label is not possible for complex products that contain 100s-1000s of different species or products that contain microbes that are not easily grown in a lab such as mycorrhizal fungi.

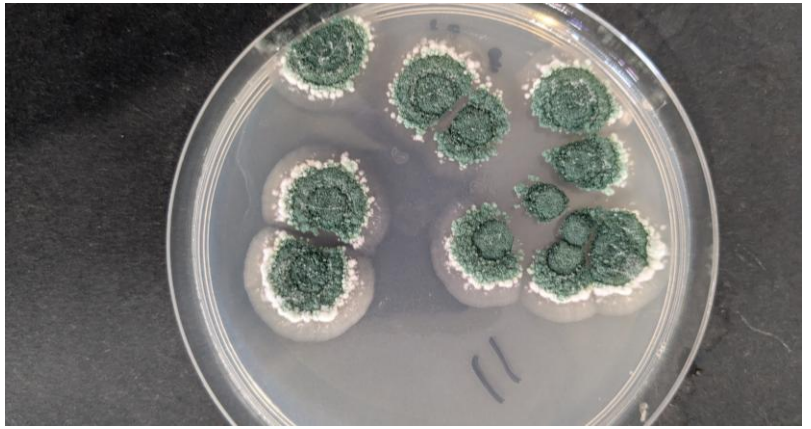
Colony forming units (CFU)???

- Bacteria and fungi can be grown in a lab on solid media known as agar plates.

Colony forming units (CFU)

- Bacteria and fungi can be grown in a lab on solid media known as agar plates.
- After a few days once the microbes have had some time to grow, each single cell forms visible mass of cells on the agar known as a colony.

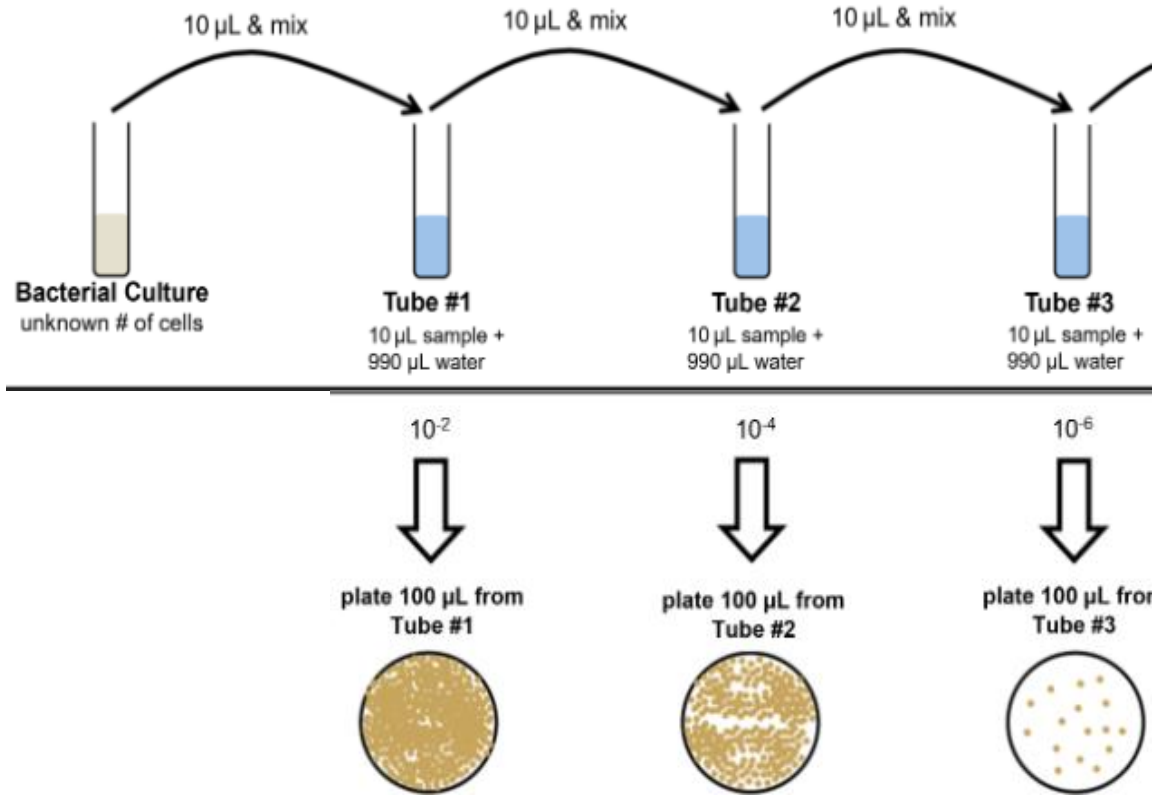
Bacterial and Fungal Colonies



Colony forming units (CFU)

- By adding dilutions of a product to an agar plate we **can grow the microbes**, **count the colonies** and **determine the concentration (CFU/ml or g) in a product**.

- DNA sequencing done on a colony to confirm the species.



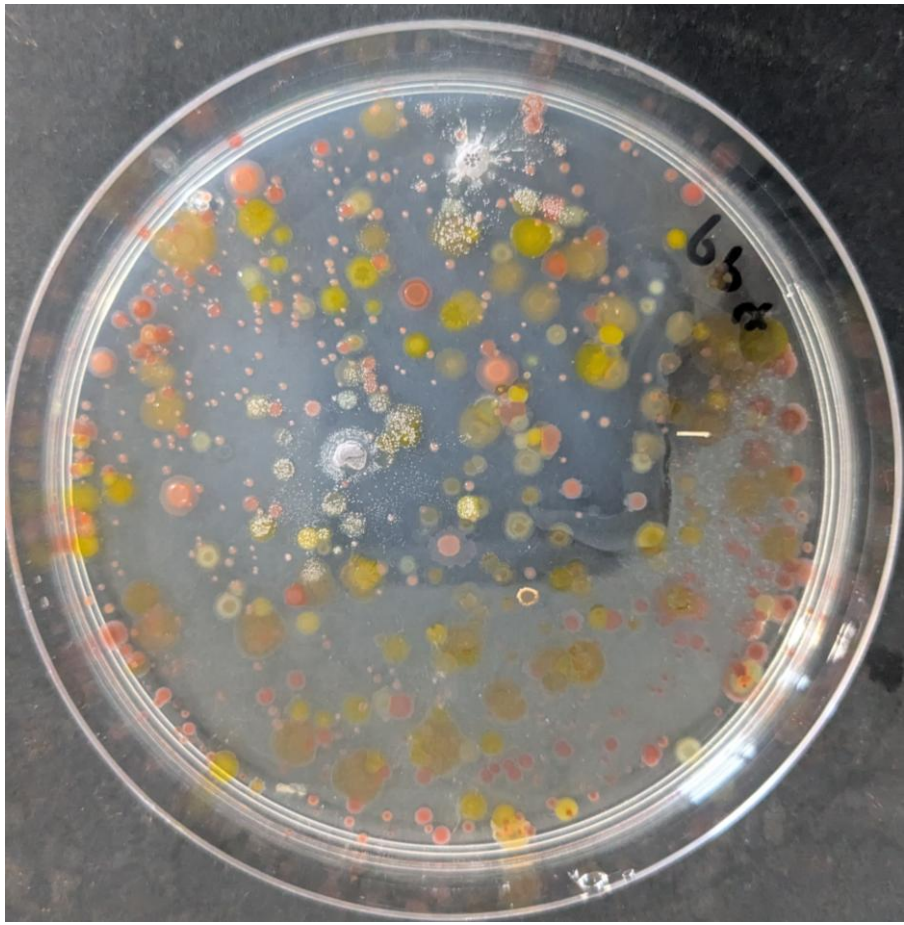
- This is the main method used in microbiology as it only counts living cells.

- This was the main method that was used for analysing products for this project.

- Works well for single species products but becomes trickier for multi species live microbial products.

Why is CFU not possible for more complex products?

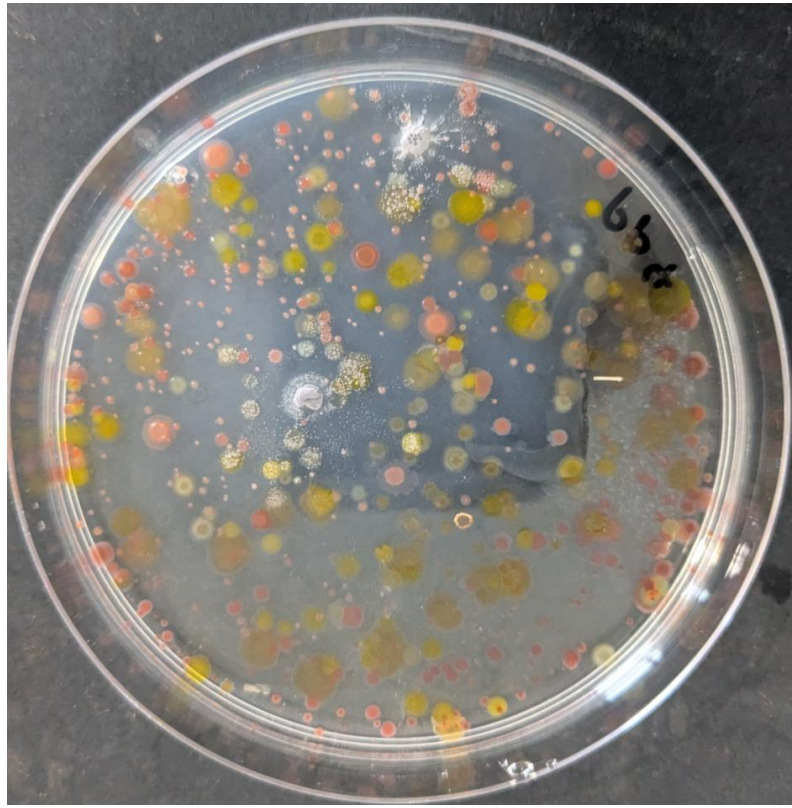
- Some microbes produce antibiotics interfering with the growth of each other.
- Some microbes require specific agar.
- What do we count here? Red ones, yellow ones, small ones, big ones?



DNA barcoding: a method for more complex products.

- In this instance DNA barcoding becomes a better way to investigate what microbes are in a product. The limitation is that you will detect DNA from dead organisms.

Extract DNA from complex product → DNA barcoding → Profile of microbes and abundances present in product



- For products where CFU counting did not work to well we did DNA barcoding to see if we could validate what was in them.

DNA barcoding results example

Out of all DNA detected 47% of it was Rhizobium for product d

	A	B	C	D	E	F	G	H	I	J
1	product a	product b	product c	product d	Genus					
2	22.23937	21.41179	33.17918	47.15263	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium					
3	18.94683	18.78659	16.33569	0	Massilia					
4	7.691177	12.52937	11.93326	0	Aquabacterium					
5	7.198765	7.40575	1.006529	0						
6	4.821225	5.32498	4.19387	0	Mucilaginibacter					
7	3.318267	1.875676	2.83823	1.303976	Bacillus					
8	2.936097	4.381549	5.336416	0	Curvibacter					
9	2.884651	3.124883	2.375771	0	Novosphingobium					

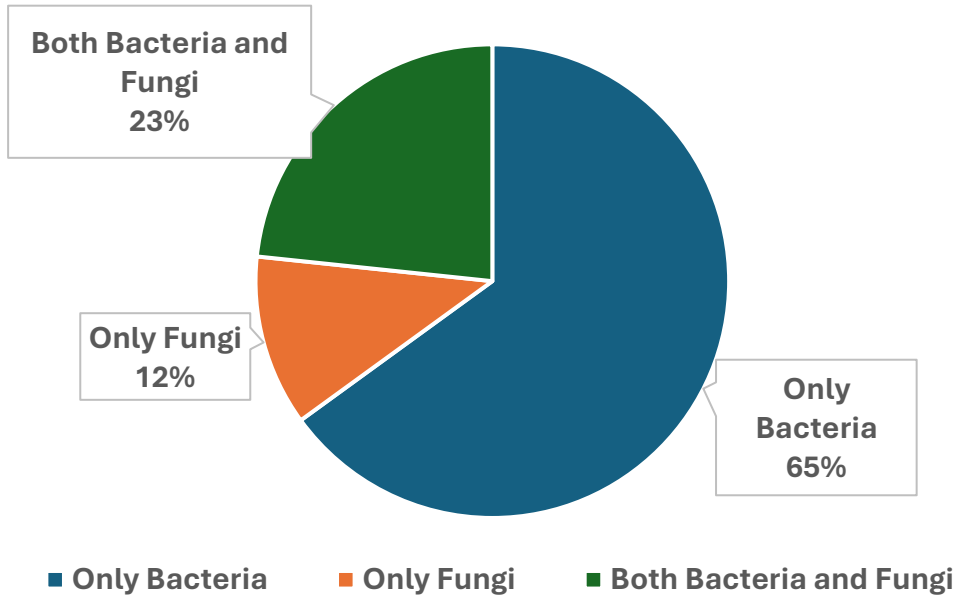
University of Queensland Masters Project Results

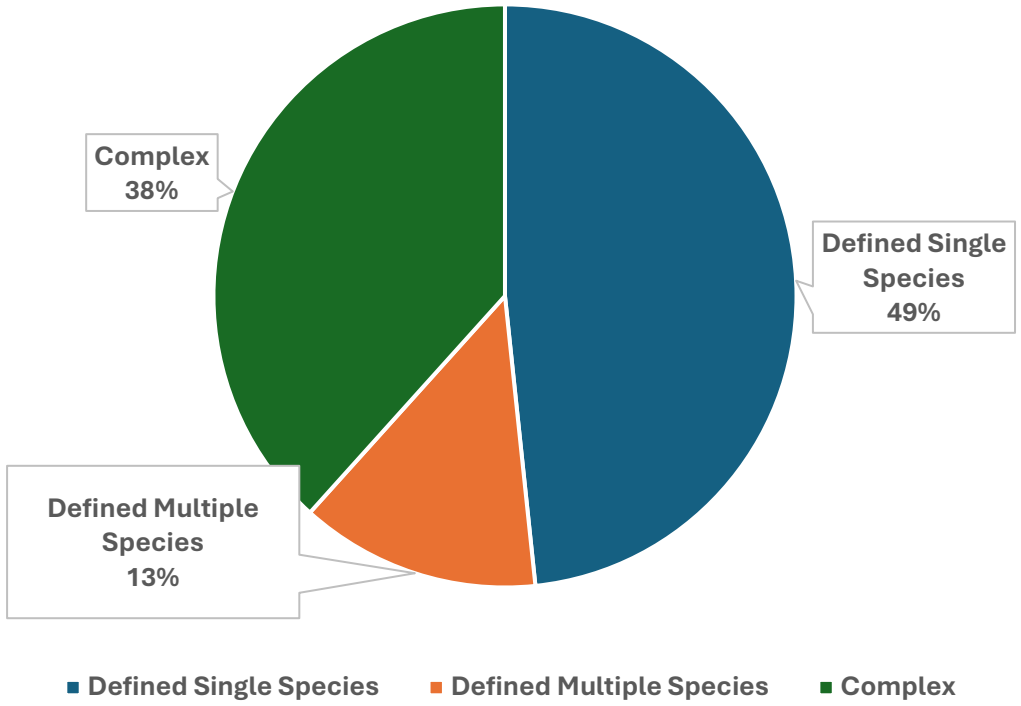
Rohit Krishna, Bachelor and Masters of Ag science, University of Queensland Gatton.

So what did we find???

Summary of products sent

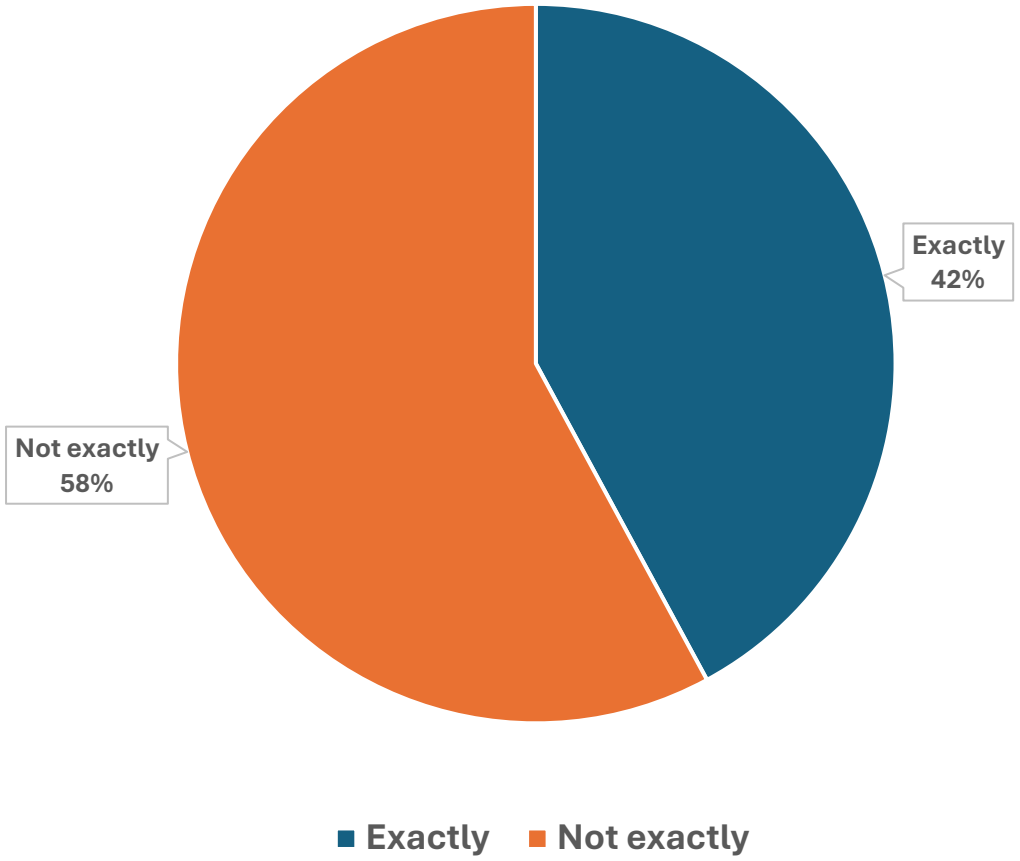
- Tested **64 products** from **19 people** covering **30 microbial species**.





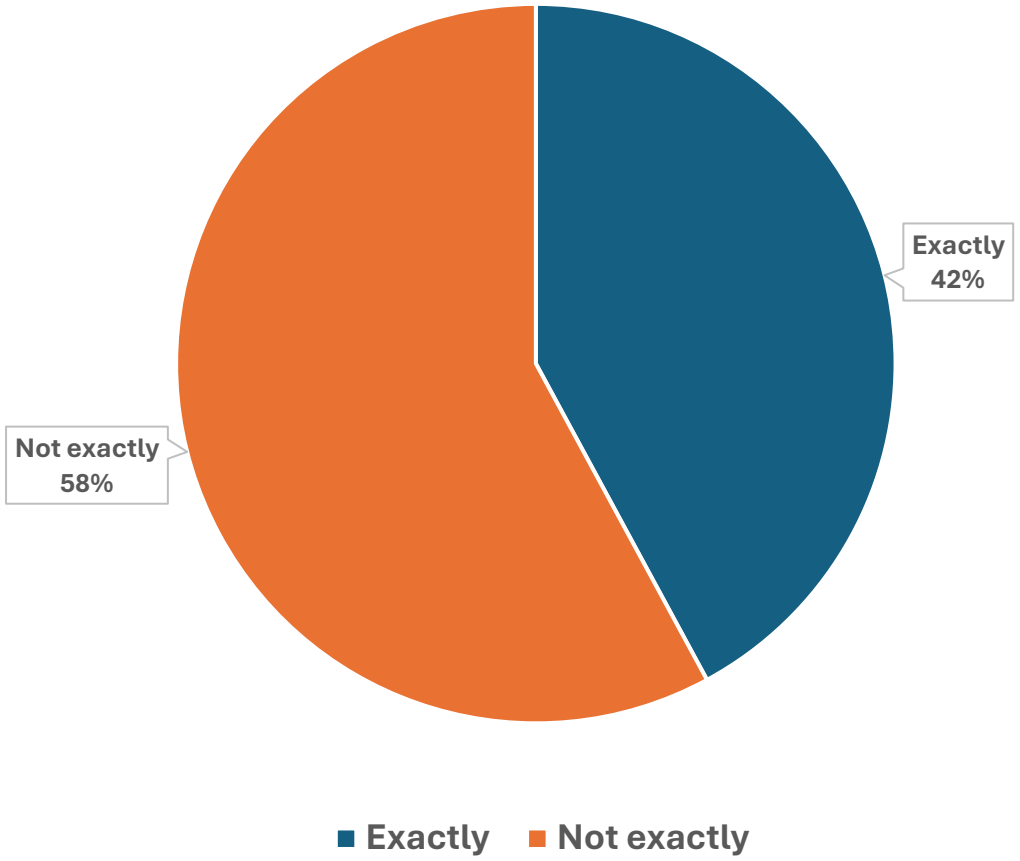
The kinds of products we tested were mostly defined single species and mostly bacterial products.

Overall results



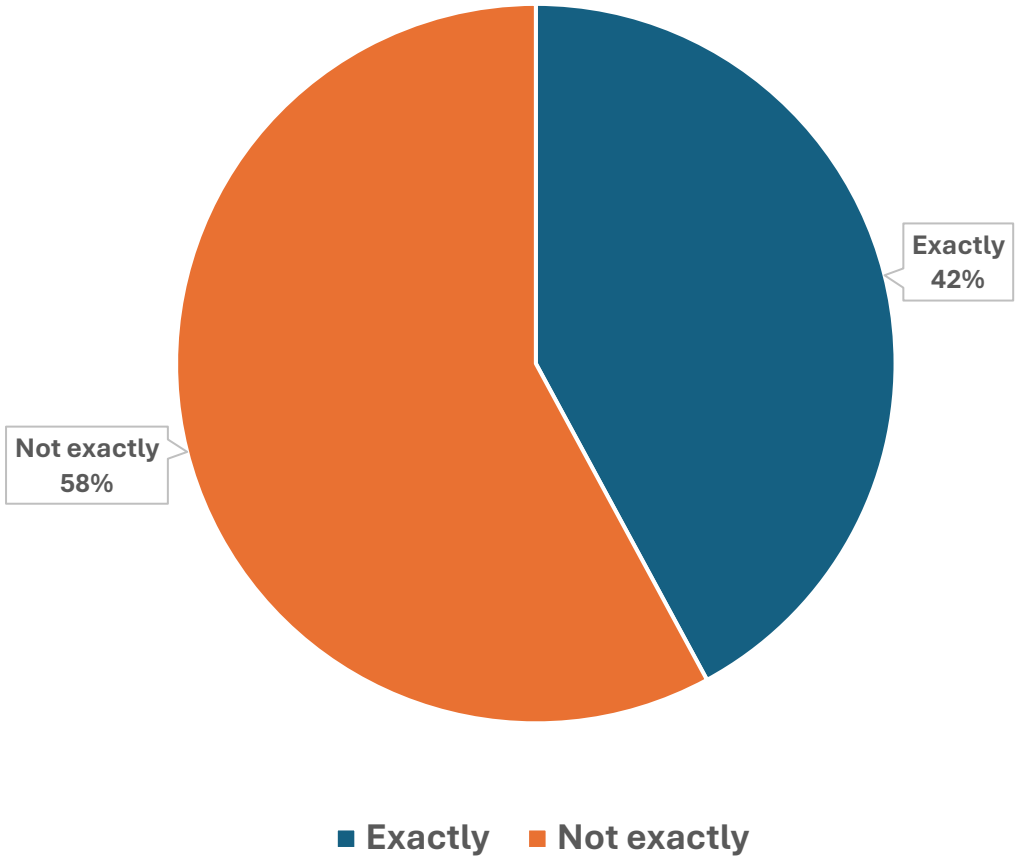
- 42% contained exactly what we were told to expect.

Overall results



- 42% contained exactly what we were told to expect.
- 58% did not contain exactly what we were told to expect.

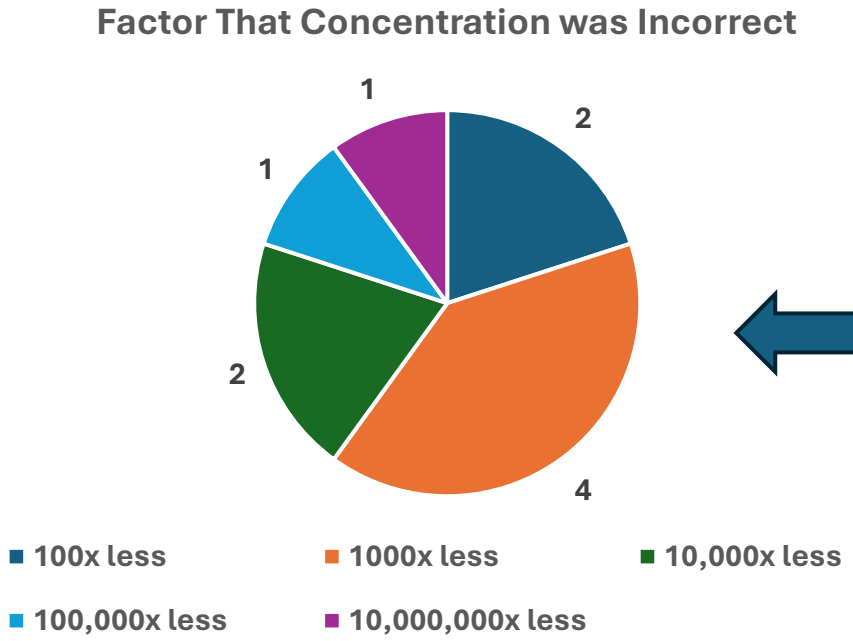
Overall results

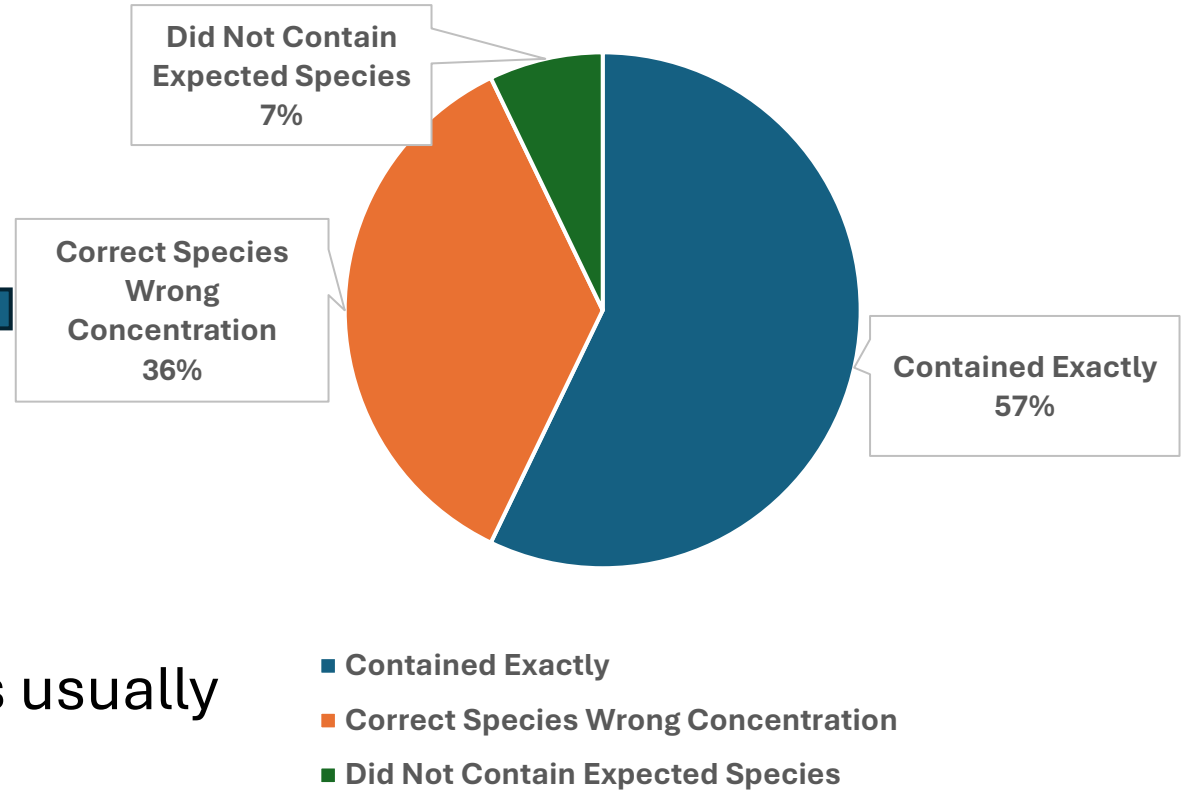


- 42% contained exactly what we were told to expect.
- 58% did not contain exactly what we were told to expect.
- However, to really understand the results we need to look a bit deeper at the individual product categories.

Defined single species products

- Most single species products did contain exactly what was expected (57%) or at least the expected species but the wrong CFU (36%).
- Only 7% did not contain the expected species.

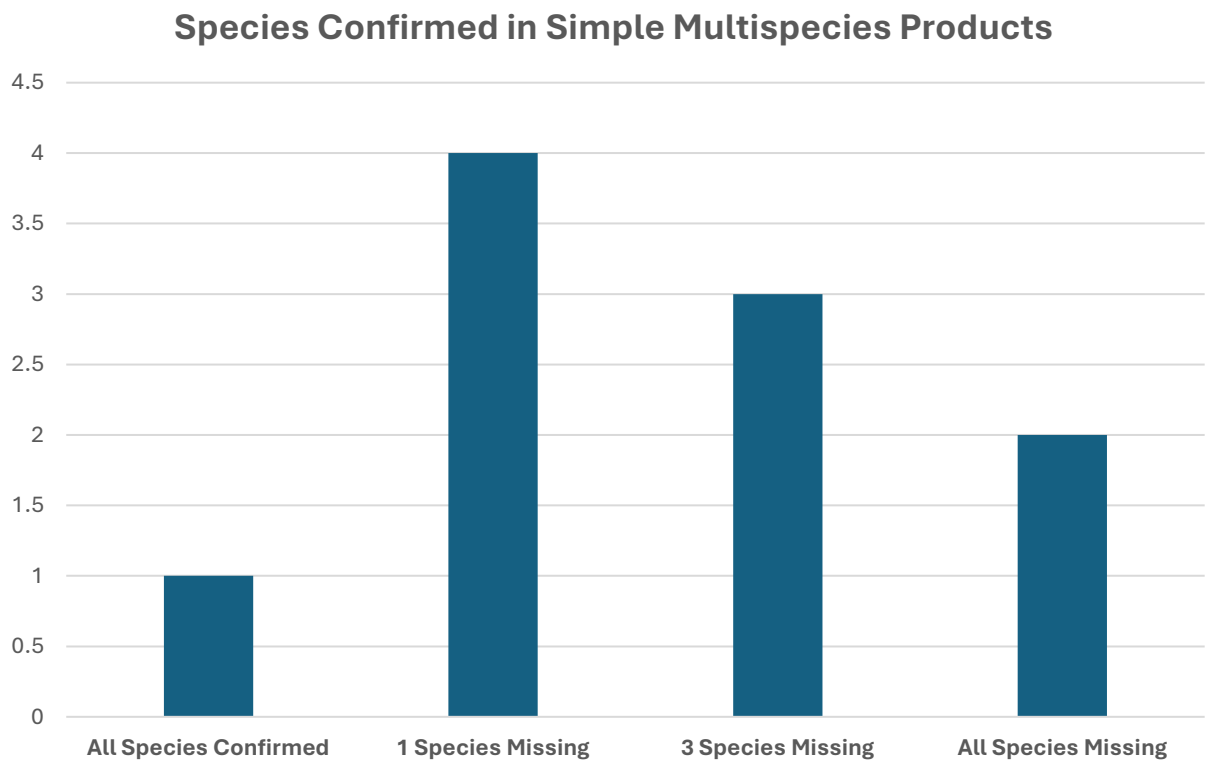




- When the concentration was incorrect it was usually 100x less – 10,000x less CFU.

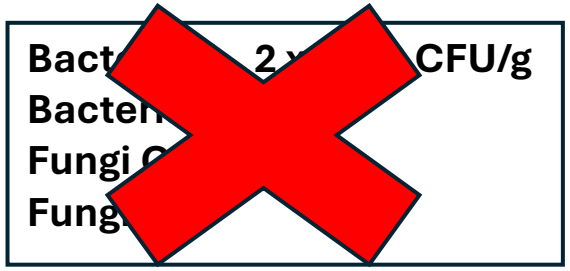
Defined multispecies products

- Only 10 products in this category were tested. Only **one** contained the multiple species expected but was 1000x lower CFU.
- 4 products missing at least 1 species, 3 missing 3 species , 2 missing all expected species



- Most of these products were not supplied with an expected CFU but for the ones that did the CFU we found did not match what we were told to expect.
- From these results it seems like multispecies products are less likely to contain exactly what is expected in them compared to single species products.

Example of poor labelling for multispecies products and a better practice



- For several products we were told to expect something like this. There are a few issues with labelling a product like this.
- From a formulation perspective it is very unlikely that you can get the exact same concentration of multiple species in a product.

Bacteria A	**2 x 10^9 CFU/g**
Bacteria B	**2 x 10^ 5 CFU/g**
Fungi C	**4x10^6 CFU/g**
Fungi D	**4x 10^7 CFU/g**

- Something like this would be more realistic.
- Again, this is the kind of thing the code of practice will help address.

Challenges with labelling and validating complex live microbial products.

What were told to expect:
Contains 13+ selected microbial species.

- Very hard to validate something like this.
- Very unlikely that some of these microbes don't out compete others in the product.

Results:

- 38 different bacterial DNA sequences detected overall. Most at very low amounts and are likely contaminants.
- Over 90% of the bacterial DNA detected belongs to two different bacterial species.

product	Kingdom	Phylum	Class	Order	Family	Genus
47.33	Bacteria	Proteobac	Gammapr	Betaprote	Burkholde	Burkholderia
42.78	Bacteria	Proteobac	Gammapr	Xanthomo	Rhodanobacteraceae	
6.23	Bacteria	Proteobac	Gammapr	Xanthomo	Rhodanob	Frateuria
1.05	Bacteria	Proteobac	Alphaprot	Sphingomc	Sphingomc	Sphingomonas

Challenges with labelling and validating complex live microbial products.

What we were told to expect:
Azotobacter vineladii, Bacillus cereus / thuringienis, Bacillus subtillis, Bradyrhizobium japonicum, Chaetomium globosum, Endo & Ecto Mycorrhiza, Fusarium spp, Nematodes, Ochrobactrumanthropi, Protozoa – flagellates, amoebae, ciliates, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas pseudoalcaligenesl, Pseudomonas putida, Pseudomonas stutzeri, Rhizobium leguminosarum, Sphingomonas paucimobillis, Streptomyces albidoflavus, Streptomyces celluosae, Trichoderma lignorum

- There is no way this product contains the exact same microbes every time it is made.

Results:
- 47 unique Eukaryotic DNA sequences detected but 98% was from a type of protozoa.
- 448 unique bacterial DNA sequences detected.
- Some of the above were detected and some were not. While some of what we detected are not mentioned above.

Challenges with labelling and validating complex live microbial products.

These labels do not mean these are bad products.

- It just highlights the challenges we face trying to label and validate complex live microbial products.

Conclusions

- **When testing defined single species products we mostly found exactly what was expected but occasionally lower CFUs then expected.**
- **There were more issues with multispecies products. Usually, could not confirm all the expected species were present and when we did the CFU was lower than expected.**
- **Complex multispecies products have their own unique challenges around labelling and validating them but that does not make them bad products.**

Questions and whats next?

- We have the fertilizer code of practice to guide labelling.
- We have the tools to test products and validate the labels.
- We have shown that products do not always contain what is expected or what is on the label.

- **So how do we take what we have learned here and move forward as an industry to have more robust and credible products?**

- **Is there a way for these tools to be implemented to ensure these kinds of products are tested before going to market?**

- **What would everyone here like to see done to improve this space?**