FOR BETTER FOOD & HEALTH

Microbial Contaminants Relevant to Safety and Quality of Plant Protein-Based Dairy Alternatives

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NIZO, Ede, The Netherlands

- Independent company
- Confidential contract research for the food industry
- Roots in dairy, > 15 years non-dairy

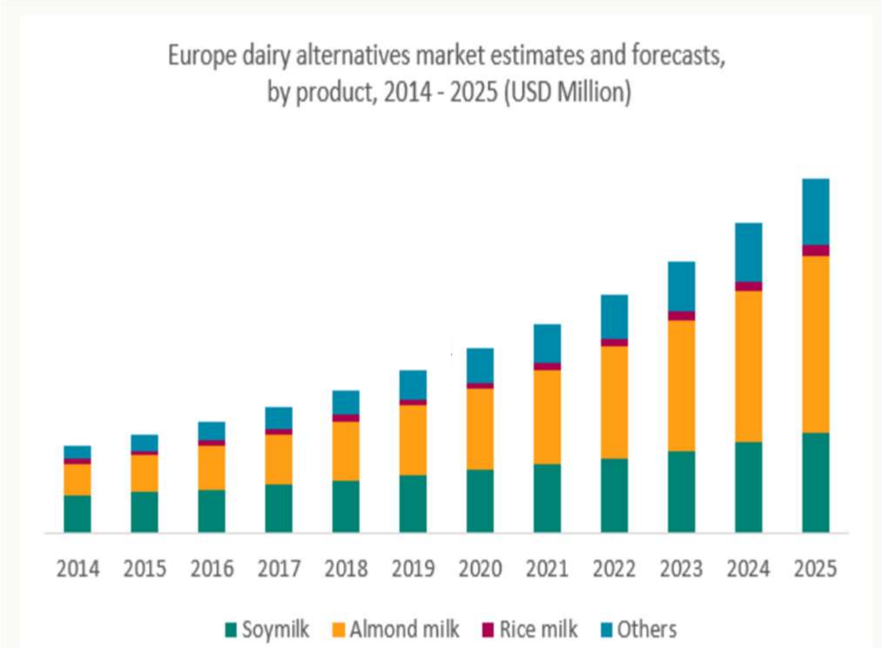
Our markets

Market trends

- Increasing demand for plant-based products

- Consumer’s choice for dairy alternatives:

    - Casein allergy / lactose intolerance
    - Vegan, no animal products
    - Sustainability – reduce ecological footprint



https://www.vegansociety.com/news/market-insights/dairy-alternative-market/european-plant-milk-market

Plant protein based alternatives

- Making a nutritious, tasty product with the right texture
  - Ingredient / composition choice

- Product safety
  - Allergens
  - Chemical contaminants
  - **Microbiological contaminants**
    - throughout the chain and in finished product

Dairy products – many decades of know-how

- Control of microbial contaminants – product and process specific
  - Levels and types of contaminants in raw milk
  - Processing contaminants

Farm

Feed, environment, milking installation

Factory

Control measures in place (GMP, HACCP)

Product

Pathogens / Spoilers

Plant-based ingredients

many sources, production processes, and qualities

Class or category (biological origin)		Protein sources
Legumes	Starch containing	Pea, faba bean, chickpea, lentil, mung bean
	Oil containing	Soy, lupin
Cereals		Oat, wheat, barley, rice, quinoa, amaranth
Nuts		Hazelnut
Stone fruit		Almond, coconut
Oil seeds		Canola, rapeseed, sunflower, hemp, flax





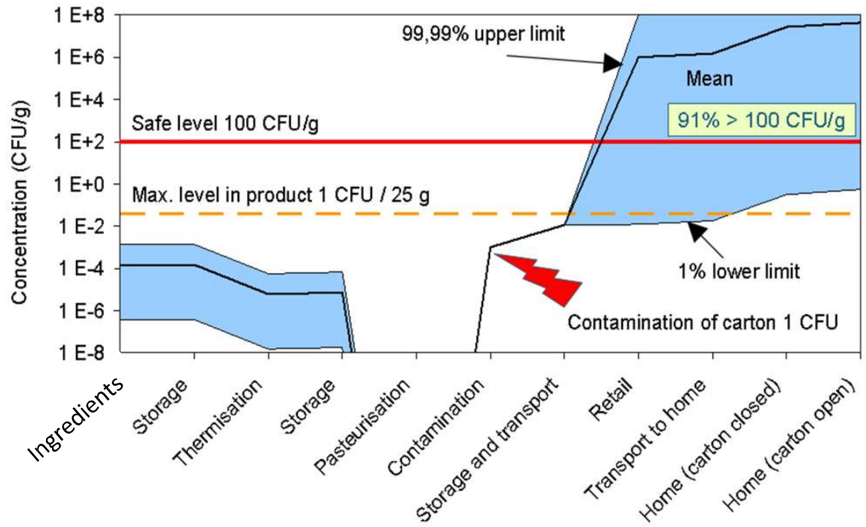

Dairy alternatives

different challenges for different product categories

- Chain control to ensure microbial safety / quality

- Microbes in ingredients – what can be expected?
  - Levels and types?
- Product specific (drinks/yogurt-/cheese-like)
  - Heat treatments – what can be applied for products?
  - Post processing contamination?
  - Does product support growth of species present?
  - Storage conditions

Quantitative risk assessments for target microbes



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Establishing microbes in different plant proteins

- Many different ingredients investigated:

- Different qualities
  - Flour, protein concentrate, protein isolate, kernels, flakes, hydrolysates

- Ingredients typically undergo heat treatment (at least 80°C)

- What are the levels?
  - Aerobic mesophilic plate count
  - Different groups of spore formers

- What are the types?
  - Isolates from highest dilutions: most prevalent species ID-ed

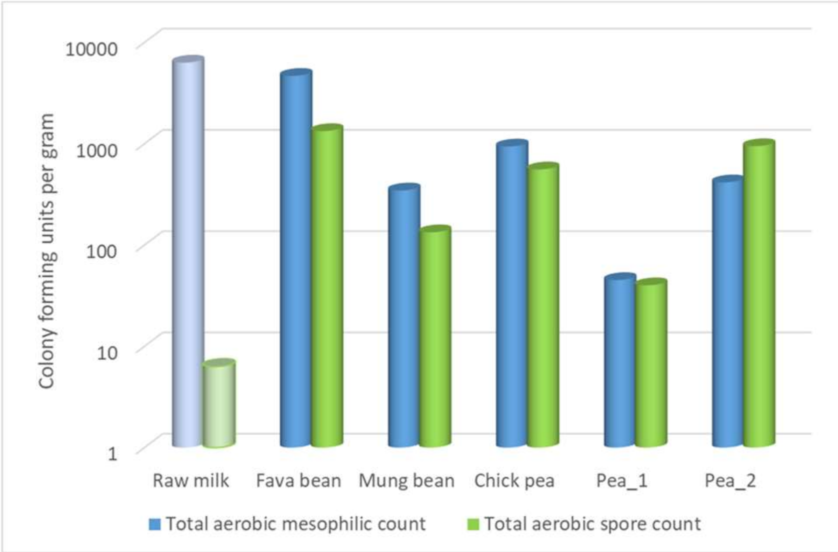
Determining contamination levels in different plant proteins

- Pea, faba bean, mung bean and chickpea
  - Flours
  - Isolates
  - Concentrates

- Analysis
  - Aerobic mesophilic plate count
  - Spore formers - aerobic mesophilic, thermophilic

Conclusion

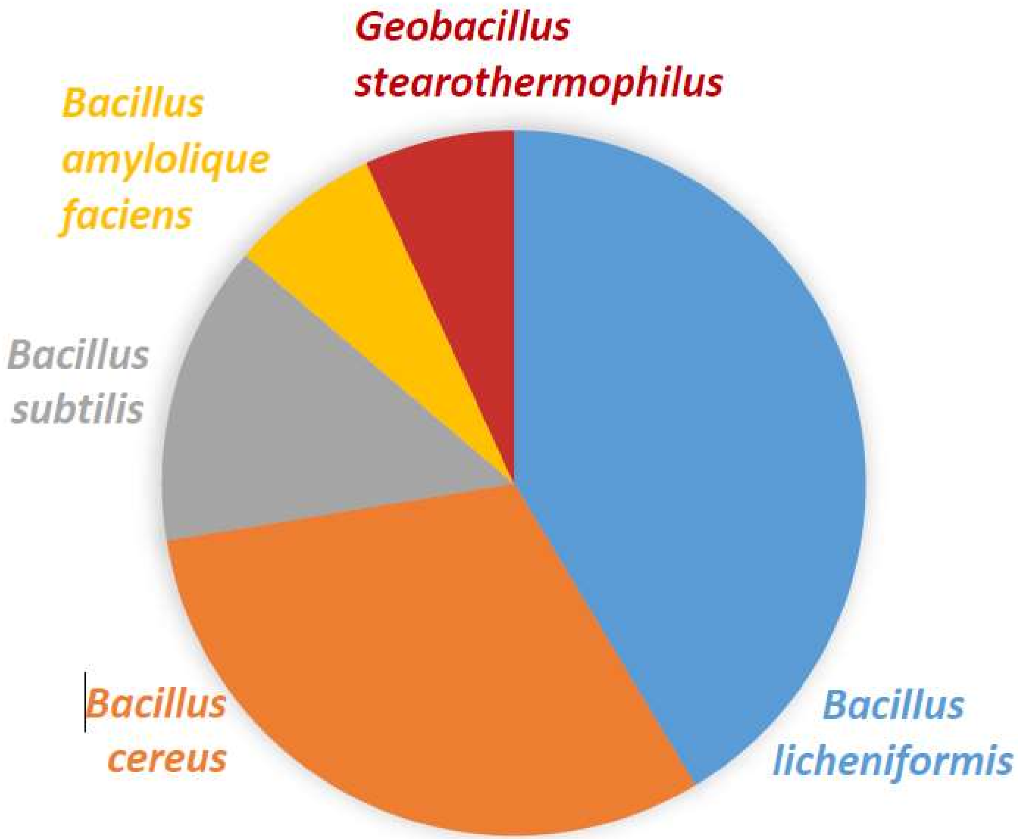
- Plant protein ingredients:
  A significant fraction of the total count consists of spores (up to ~ 3 $\log_{10}$ /g)



Total aerobic counts and mesophilic spore counts in samples of raw milk and various plant protein isolates (fava, mung bean, chick pea, pea)

Determining species present

- Representative isolates from the highest dilutions were identified
- Predominant species
  - *Bacillus subtilis, B. licheniformis, B. amyloliquefaciens*
  - *Bacillus cereus*
- Also present:
  - Sulfite reducing clostridia (SRCs)
  - *Geobacillus stearothermophilus*



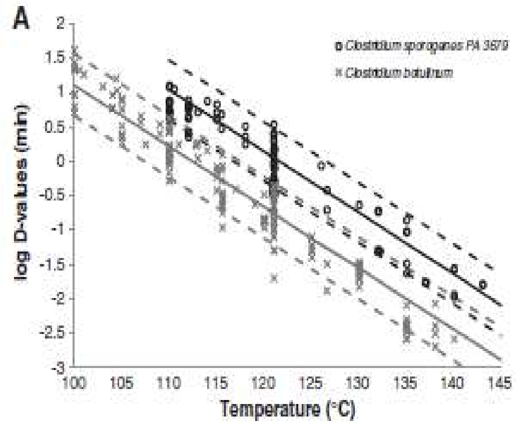
Drinks

- Ensure sterility of drinks - thermal processing

- Calculation heat treatment
  - Initial levels and types
  - Apply optimal thermal process for inactivation

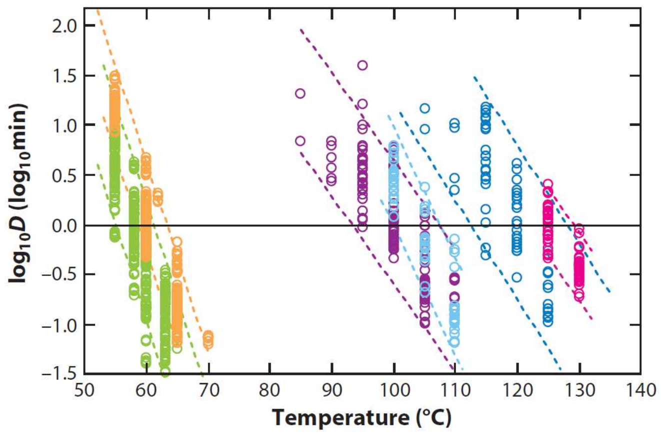
- Some spores survive high heat treatments (UHT)
  - surviving thermoresistant spores
    - *Geobacillus* spp. (storage >45°C)
    - *B. subtilis* isolates with genetic elements that render high level heat resistant spores (ambient stable)

Inactivation spores *C. botulinum* (I) and *C. sporogenes*



Diao et al 2014

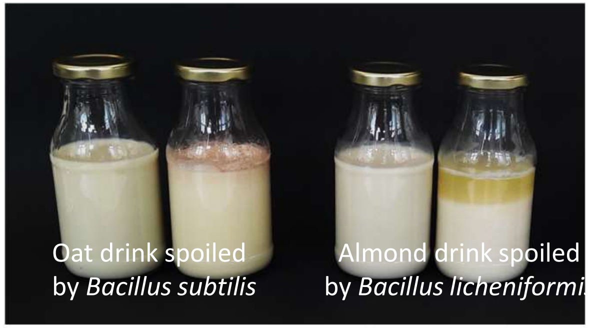
Inactivation of bacteria and spores of *B cereus, subtilis, Geobacillus*



Den Besten et al 2018

Drinks - neutral pH, UHT – non-sterility issues

- Commercial non-sterility:
  - One spore per packaging unit is enough!
- Spoilage by:
  - surviving thermoresistant spores
  - contamination in installations / fouling issues
    - Limited protein solubility / denaturation
    - *B. subtilis / licheniformis / circulans / cereus*
- Food safety risk in case of
  - insufficient heating *C. botulinum / B. cereus*
  - fouling *B. cereus*



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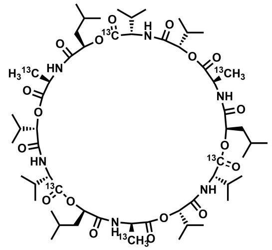
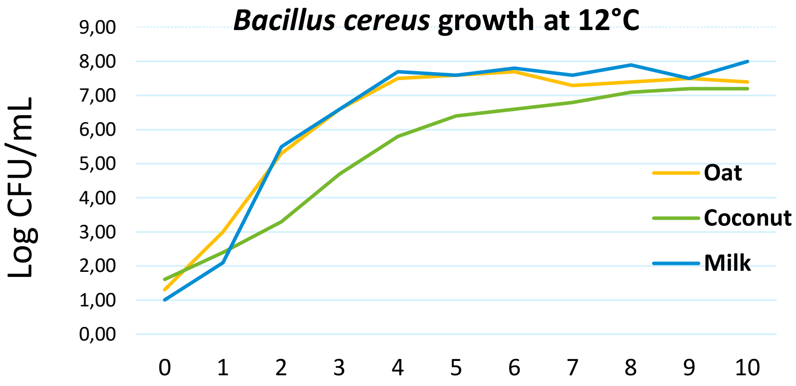
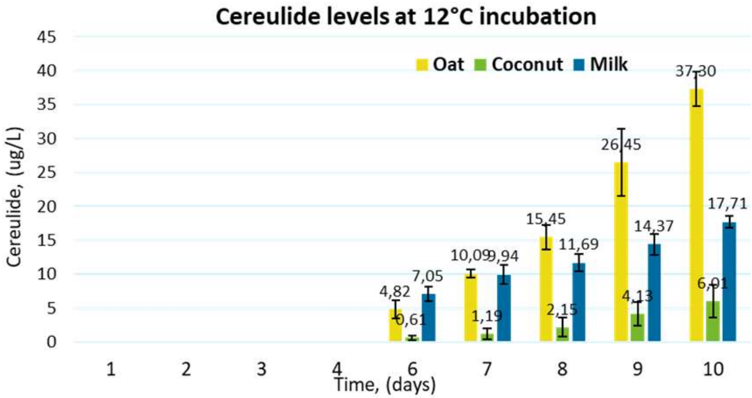
Oatly recalls drink because of Bacillus cereus; 2 sick with 27 other complaints filed

Bacillus cereus and cereulide production in plant based substrates







- Production of cereulide after 6 days at 12°C, after 12-16h at 30°C

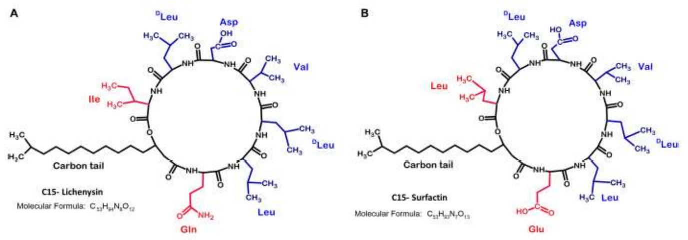
Surfactants by *B. subtilis* and *B. licheniformis*

- Lichenysin and surfactin from *Bacillus* spp
  - Highly heat stable (121°C, 20 min)
  - Lipopeptide, Non-ribosomally synthesized
  - Gene cluster: *srf* surfactin, *lic/lchA* lichenysin

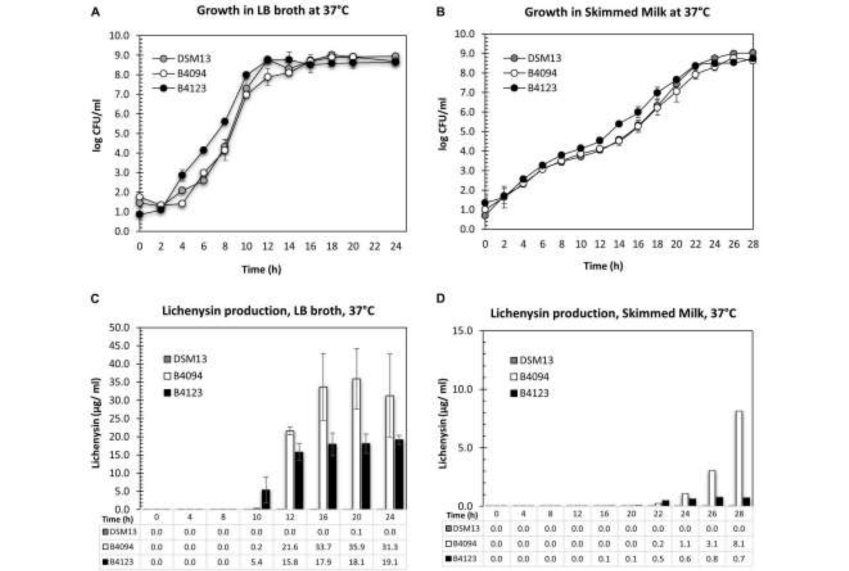
- Isolates from plant ingredients: *lic/lchA* +

- Lichenysin production:
  - late exponential phase / high cell density locally
  - not detectable under 10^5 CFU/mL in milk and LB
  - Levels in LB relevant (16 ug/mL toxic to Caco-2)

- Production on plant substrates?



Lichenysin production by *B. licheniformis* in LB and milk
Yeak et al. *Frontiers in Microbiology*, 2022



Time (h)	0	4	8	10	12	16	20	24
DSM13	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
B4094	0.0	0.0	0.0	0.2	21.6	33.7	35.9	31.3
B4123	0.0	0.0	0.0	5.4	15.8	17.9	18.1	19.1

Time (h)	0	4	8	12	16	20	22	24	26	28
DSM13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B4094	0.0	0.0	0.0	0.0	0.0	0.0	0.2	1.1	3.1	8.1
B4123	0.0	0.0	0.0	0.0	0.1	0.1	0.5	0.6	0.8	0.7

FIGURE 3 | Growth and lichenysin production for DSM13^T, B4094, and B4123 in LB and skimmed milk at 37°C. **(A)** Growth of three selected strains, DSM13^T, B4094, and B4123 in LB medium for 24 h. **(B)** Growth of three selected strains, DSM13^T, B4094, and B4123 in skimmed milk for 28 h. **(C)** Total lichenysin production (µg/ml) in LB broth for DSM13^T, B4094, and B4123. **(D)** Total lichenysin production (µg/ml) in skimmed milk for DSM13^T, B4094, and B4123. The amount of lichenysin produced by all three strains in LB at selected time points as quantified with RP-HPLC-QTOF-ESI/MS. Gray circle or bar- DSM13^T, white circle or bar- B4094, black circle or bar- B4123.

Yogurt-like products

- Large variety commercially available, many at product development stage

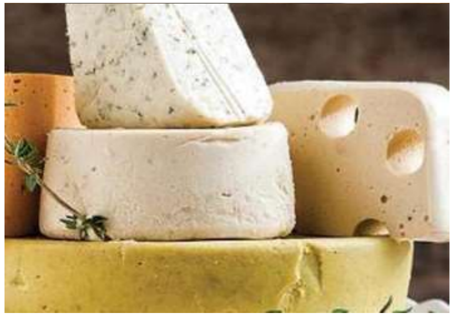
- Ingredients pasteurized
  - Generally < 95 °C
  - Yeast, moulds, vegetative cells killed (e.g. *Listeria, Salmonella)*
  - Bacterial spores survive

- Target pH below 4.5
  - acidification during fermentation by starter culture lactic acid bacteria

- Microbiological stability of fermented yogurt-like products
  - pH 4.5 + lactate present: most bacterial contaminants controlled
  - Yeast and moulds can grow – prevent contamination

Semi-hard and Cream cheese-like products

- A range of ingredients can be applied
  - based on starch
  - based on protein
- Target pH values typically 5.0 to 5.5
  - chemical acidification
  - fermentation using starter cultures
- Ingredients heated
  - yeast and moulds and vegetative bacteria inactivated
  - spores survive – *Clostridium* and *Bacillus* spp
  - post-processing contamination – slicing, extra additions?
- Potential risks:
  - Overgrowth starter during fermentation
  - Outgrowth contaminants during storage incl. post processing



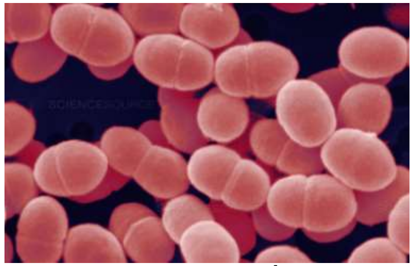
Low pH products: yogurt and cheese

fermentation with lactic acid bacteria

- Fermentation of dairy
  - Dairy starter cultures – evolved in traditional milk-based fermentation
  - Conversion of milk sugar lactose -> lactic acid
  - Development of flavour and texture, antimicrobials

- Fermentation plant based substrates
  - Different substates in plant based fermentations
  - Growth and performance different?

Streptococcus thermophilus



Lactococcus lactis

Bacillus

Acidification rates are LAB strain dependent

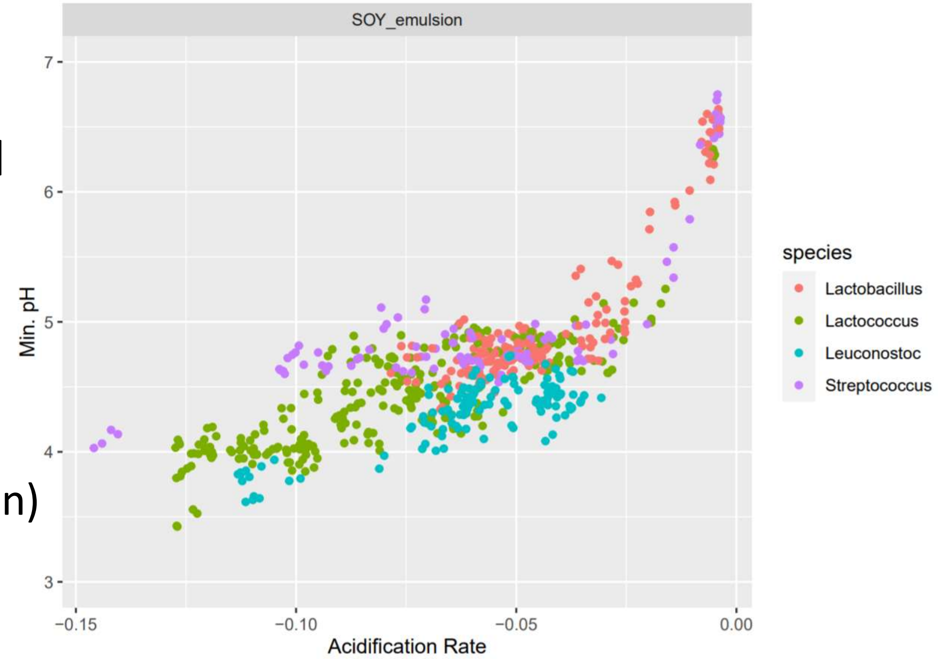
impact on food safety / spoilage

- Fast acidification and low pH important to prevent outgrowth of spoilage organisms

- Slow acidification comes with higher final pH

- Potential contaminant issues:
  - spore formers grow to pH of 4.6, some lower
  - *B. cereus* (cereulide toxin)
  - *B. subtilis* and *B. licheniformis* (surfactin, lichenysin)
  - *Clostridium*?

- Post processing contamination
  - E.g. *Listeria monocytogenes*



Fermentation of pea-protein-enriched emulsion

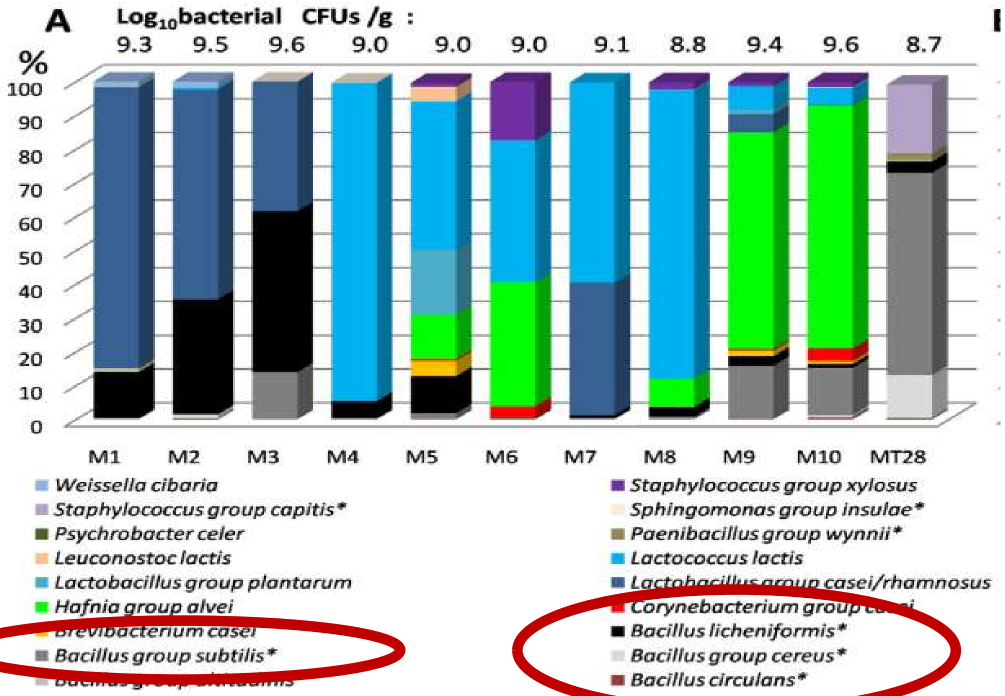
sporeformers in ingredients

- Heat treatments of pea protein isolate
  - 100°C for 30 min + 95°C for 30 min
  - Higher heating not applied to maintain protein functionality

- Fermentation with 10 different consortia

- In various samples *Bacillus* spp came up during fermentation, incl *B. cereus*
  - Spores not inactivation

- Potential risk
  - Overgrowth of starter culture



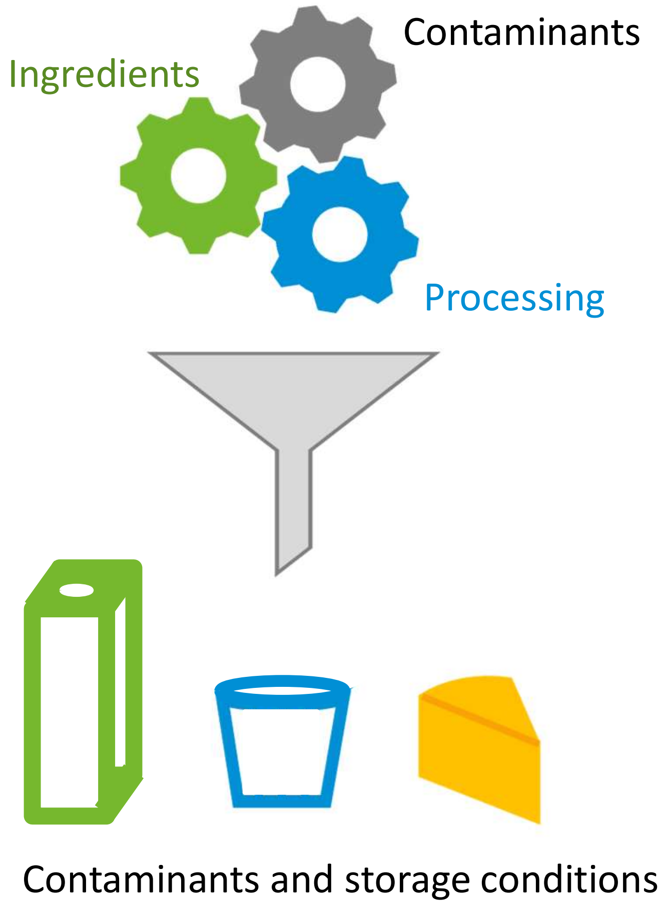
Ben-Harb et al. 2019. Int. J. Food Micro. 124.

Conclusion - Chain Control plant based products

Microbiological safety, preservation and shelf life

- Different challenges:
  - Drinks: spores (fouling/survival)
  - Yogurt: fermentation
  - Cheese: fermentation, post-processing, storage

- Control 'by design'
  - *In silico* modelling (pathogens, spoilers) to assess risks
  - Setting relevant specifications
  - Verification



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  - Fermentation
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Topsector Agri& Food TKI Agri&Food

- TKI projects
  - Plant based cheese
  - Biopurification to remove off-flavours
  - Microbial contaminants plant-based