Mejeriforskningens Dag

Bioprotective cultures

- From a scientific point of view

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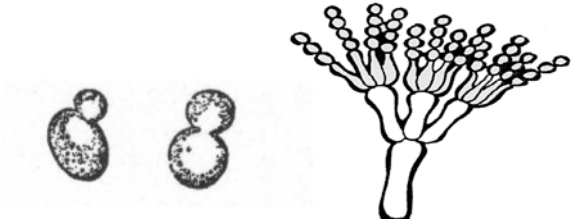
How do bioprotective cultures work

- Factors influencing inhibitory activity

The spoilage organism

Variation in concentration and sensitivity



The dairy product

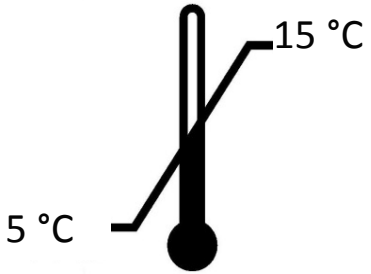
Different water activity, fat, salt, pH,…

The production of the dairy product

Fermentation time and temperature

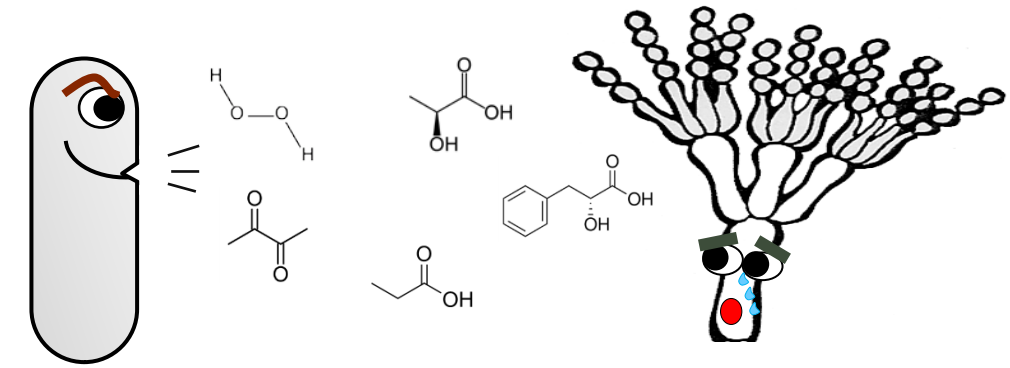
Storage of the final product

Temperature and time



What is the mode of action of bioprotective cultures?

- Fungistatic → growth of the microorganism is inhibited/delayed
- The exact mode of action is not known
- Minimal Inhibitory Concentration (MIC) is often higher than produced concentration by bacteria (synergistic effects?)



Competition for nutrients and space
Cell interaction
Cell communication

Production of organic acid
Lactic acid, acetic acid, propionic acid,…

Production of other inhibitory compounds
Proteinaceous compounds, H_2O_2, diacetyl, fatty acids, phenyllactic acid,…

Scientific literature about bioprotective cultures

Identification of new bioprotective bacteria

- Sources: Sourdough, beer, milk, cheese,…
- Bioprotective microorganisms identified
  - *Lactobacillus* sp. (many)
  - *Lactococcus lactis*
  - *Pediococcus* sp.
  - *Propionibacterium* sp.
  - …
- Active against
  - Spoilage microorganisms: yeast, molds, bacteria
  - Pathogens

Methods to study bacterial-fungal interactions

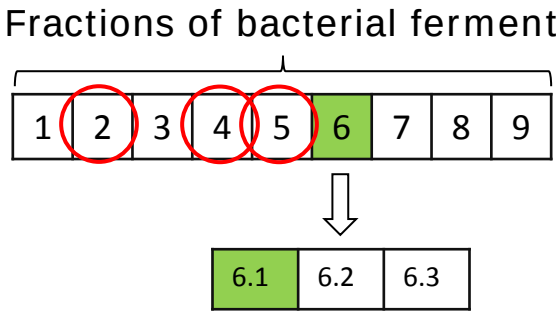
- Proteomics: Protein expression in the fungi
- Transcriptomics: Fungal gene expression
- Microscopy: Morphological changes

Identification of inhibitory compounds

- Difficult due to the complex food matrixes
- Low concentrations of produced compounds

Identifying inhibitory compounds
- Method: Bioassay-guided fractionation

- Commonly applied strategy
  - Bioassay-guidet fractionation: Targeting the inhibitory fraction
  - Suitable for single or few active compounds
  - Not suitable for combination of compounds



= Fractions show no inhibitory activity

= Combined effect: inhibitory

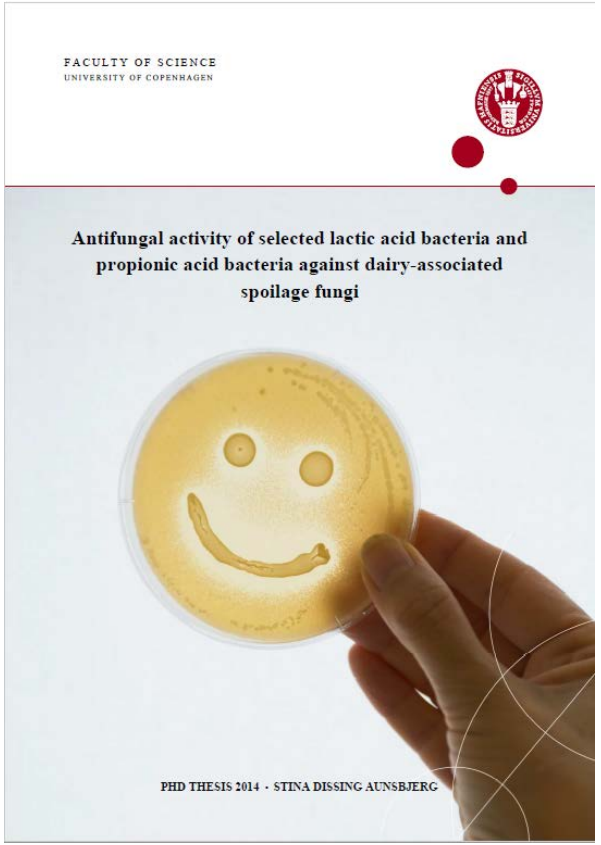
Results from my PhD project

Objectives

Investigation and characterization of the inhibitory compounds produced by *Lactobacillus paracasei* strains in yoghurt

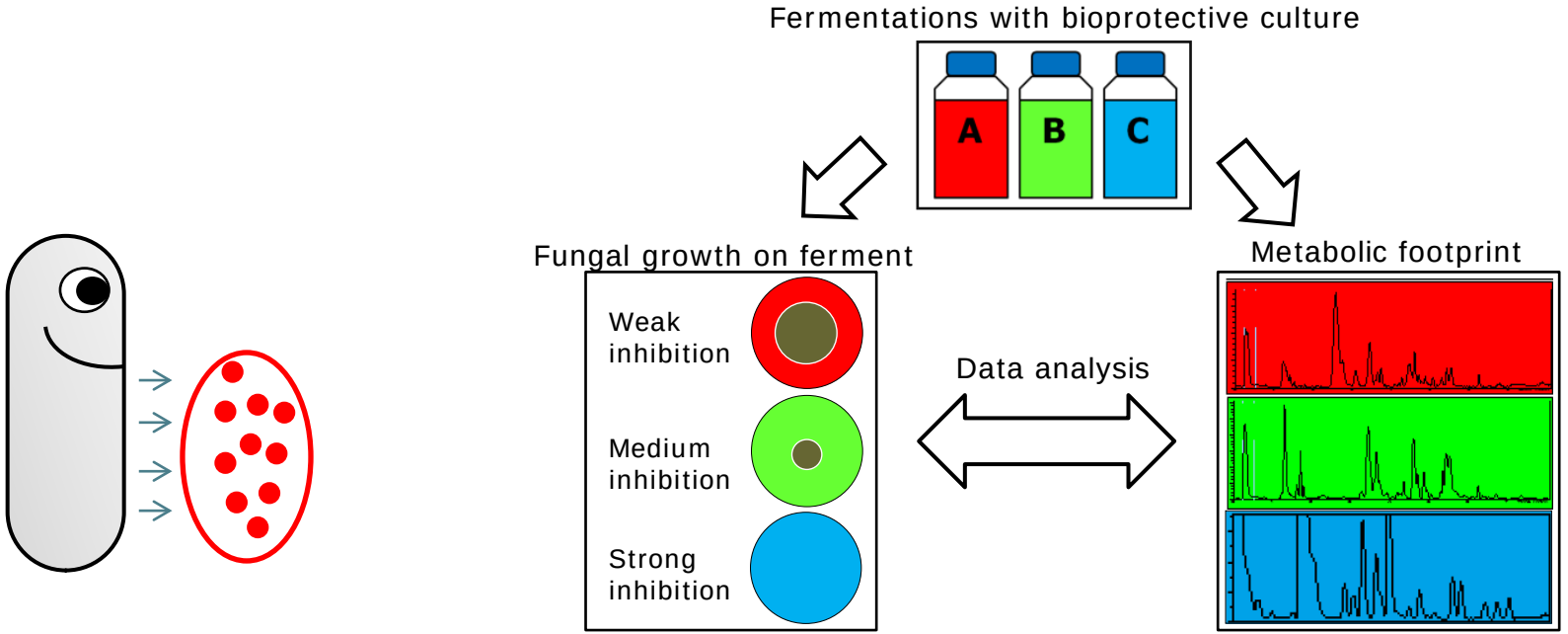
Strategy

- Use of metabolic footprinting as an alternative to bioassay guided fractionation
- A collaboration between University of Copenhagen and DuPont



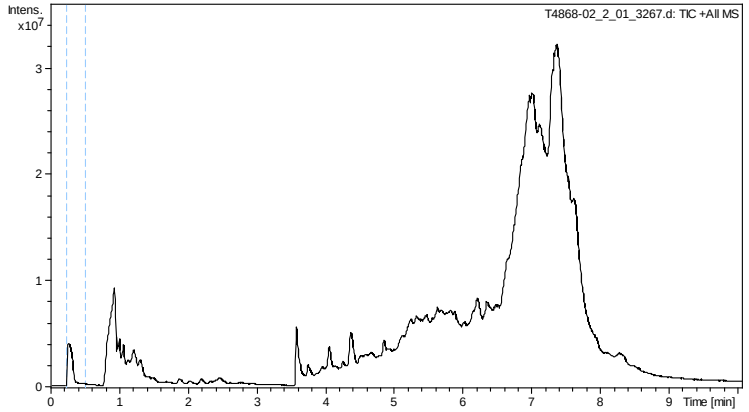
Identifying inhibitory compounds
- Method: Metabolic footprinting

- Identification of the produced compounds (inhibitory and non-inhibitory)
- Correlating inhibitory activity with produced compounds by multivariate data analysis

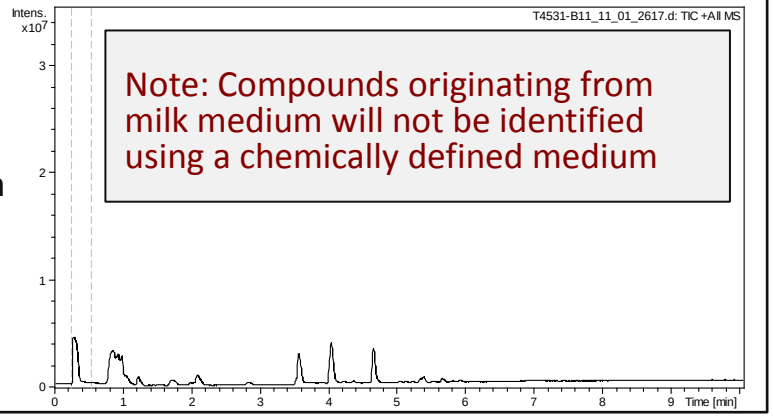


Identification of inhibitory compounds

Total Ion Chromatograms of *Lb. paracasei* **milk ferment**



Total Ion Chromatograms of *Lb. paracasei* **chemically defined medium ferment**

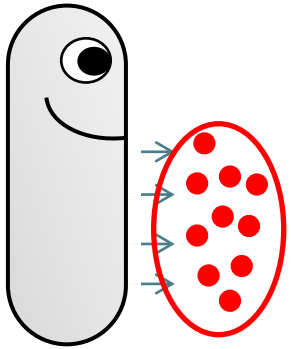


Note: Compounds originating from milk medium will not be identified using a chemically defined medium

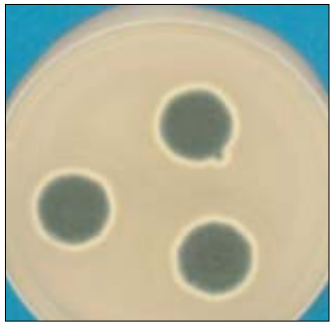
Components	LAB	PAB	Mold	Yeast	Concentration ($g\ L^{-1}$) CDIM
Glucose	x		x	x	10
K_2HPO_4	x	x	x		3
KH_2PO_4	x	x	x	x	3
$MgSO_4 \times 7H_2O$	x	x	x	x	0.5
$MnSO_4 \times H_2O$	x	x	x	x	0.1
$CoCl_2 \times 6\ H_2O$		x			0.001
$(NH_4)_2SO_4$			x	x	10
$CaCl_2 \times 2H_2O$			x	x	0.1
$ZnSO_4 \times 7H_2O$			x	x	0.01
H_3BO_3				x	0.001
NH_4NO_3		x	x		1.6
KCl			x		0.5
$CuSO_4 \times 5H_2O$			x	x	0.001
$FeSO_4 \times 7H_2O$	x	x	x	x	0.02
Adenosine		x			0.1335
Na lactate		x			16
Adenine	x				0.025
DL-alanine	x	x			0.1
L-arginine[a]	x	x			0.1
L-asparagine[a]	x	x			0.1
L-aspartic acid	x	x			0.1
L-cysteine[a]	x	x			0.1
L-glutamic acid	x	x			0.1
Glutamine	x	x			0.1
Glycine	x	x			0.1
L-histidine HCl	x	x			0.1
L-isoleucine	x	x			0.1
L-leucine	x	x			0.1
L-lysine	x	x			0.1
L-methionine HCl	x	x			0.1
L-phenylalanine	x	x			0.1
L-proline	x	x			0.1
L-serine	x	x			0.1
L-threonine	x	x			0.1
L-tryptophane	x	x			0.1
L-tyrosine[a]	x	x			0.1
L-valine	x	x			0.1
Thiamine hydrochloride	x	x	x	x	0.001
Riboflavin	x			x	0.001
Nicotinic acid	x	x		x	0.01
Calcium pantothenate	x	x		x	0.001
Pyridoxal HCl	x	x		x	0.002
Biotin	x	x	x	x	0.0001
Folic acid	x				0.0002
Cyanocobalamin	x		x	x	0.0001

Metabolic footprinting - identification of inhibitory compounds

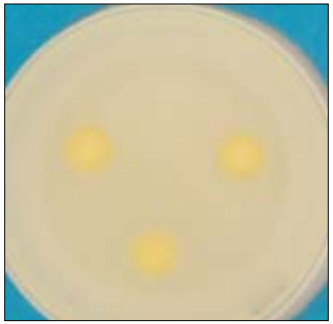
- Procedure
  - Fermentation with bioprotective bacteria in chemically defined medium
  - Centrifugation of ferment to remove bacteria
  - Identification of produced compounds using different chemical methods



Reference
No bacteria



Ferment
Not centrifuged (+bacteria)



Ferment
Centrifuged (no bacteria)

Why is antifungal activity lost in cell-free ferments?

- Influence of volatiles

Ferment or reference plate

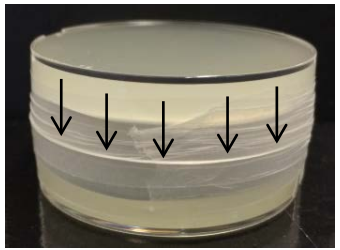
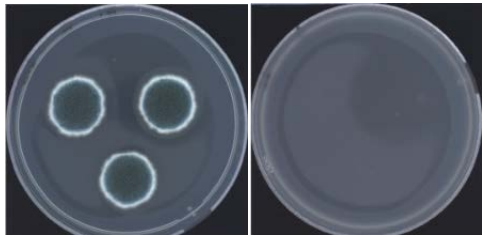


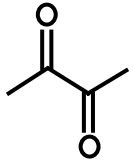
Plate with spotted mold

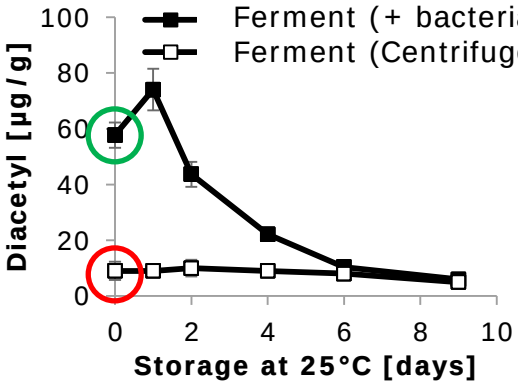
Exposed to headspace of:

Reference plate plate with ferment

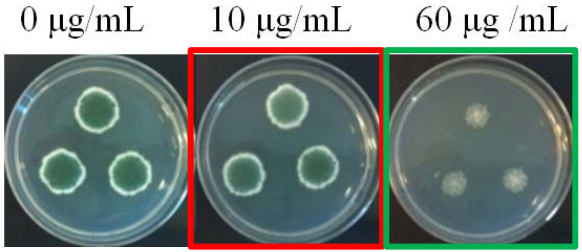


The main volatile produced: Diacetyl



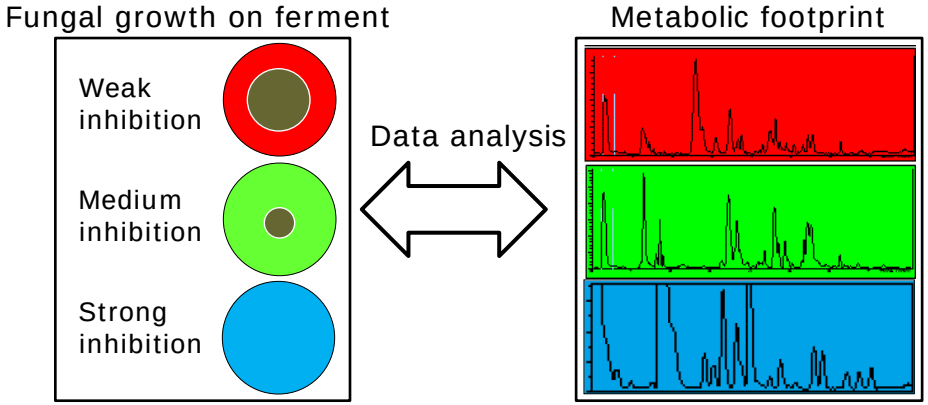


Chemically defined medium ± diacetyl (no bacteria)



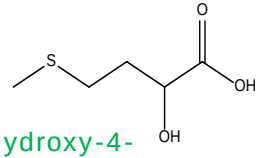
Metabolic footprinting

- *Lactobacillus paracasei* strains with varying inhibitory activity

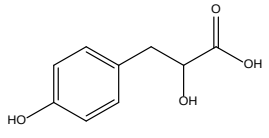


Six 2-hydroxy acids were identified:

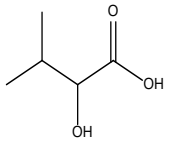
3 new and 3 previously reported in literature:



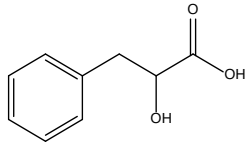
2-hydroxy-4-(methylthio)butanoic acid



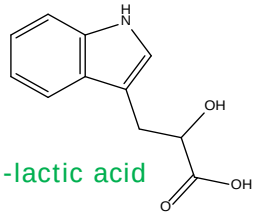
p-hydroxyphenyl-3-lactic acid



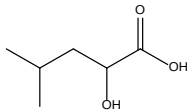
2-hydroxy-3-methylbutanoic acid



3-phenyllactic acid



Indole-3-lactic acid



2-hydroxy-4-methylpentanoic acid

All 2-hydroxy acids were antifungal but with much higher concentration needed for inhibition than the levels produced → Complex mechanism (synergistic effects?)

Continued research needed within bioprotective cultures

Finding new application areas for bioprotective cultures

- Screening for new bioprotective strains
- Find solutions for product types where bioprotection is not used today

Understanding the mode of action in the real food matrix

- Identification of produced inhibitory compounds
- Optimization of methods for identification of inhibitory compounds
- The mode of action of isolated compounds
- The role of bacterial-fungal interactions
- The role of competition for nutrients and space

Interaction with starter culture

Influence of fermentation temperature on activity

Why is research relevant for the dairy industry?

- Access to the latest research results
- Development of new competences
- Use of innovative new methods
- Dedicated students

- Knowledge about inhibitory mode of action of bioprotective cultures will allow
  - Screening for new strains
  - Entering into new application areas