Selection of effective antifungal cultures in fermented dairy products and identification of antifungal metabolites

Marcia Leyva Salas¹, Jerôme Mounier¹, Gilles Garric², Marie-Bernadette Maillard², Florence Valence², Emmanuel Coton¹, Anne Thierry²

¹Lubem, Univ Brest, Plouzané, France
²UMR STLO, Inra, Rennes, France
Déclaration de conflit d’intérêt

Pour cette présentation, je déclare n’avoir aucun conflit d’intérêt.
AUDITION Prix de thèse, 30 sept 2019

Marcia Leyva Salas
Introduction

Fungi of dairy products

Some essential fungi in dairy products...

... but others responsible for spoilage!

Impact on the organoleptic quality of dairy products
Food waste and economical losses
**Biopreservation:** « *Extension of food shelf-life and increase in food safety using natural or added microbiota and/or their antimicrobial compounds* » Stiles, 1996

- Can be used to complement preventive and control methods in *replacement of chemical preservatives* (natamycin, sorbates)
- Responds to the *strong societal demand* for more natural, less severely processed and safer products

**PROFIL project**

to develop bioprotective solutions with antifungal activity suitable for use in the dairy industry
Strategy to identify antifungal strains

Scale-up approach

from **high-throughput screening** in dairy models

A selection of strains
Commercial antifungal cultures
Several inoculation levels
→ ~7000 tested conditions

**4 fungal targets:**

- *P. commune*
- *M. racemosus*
- *G. geotrichum*
- *Y. lipolytica*

isolated from spoiled dairy products
representative of the most common dairy product spoilers

**Garnier et al., 2017 Int J Food Microbiol**

$
\text{Cheese Model} \quad \text{Yogurt}
$

$
\text{Semi hard cheese} \quad \text{Sour cream}
$

**Targeted metabolomics**

to identify the antifungal metabolites involved in the observed activity in the 4 dairy products
Introduction

Previous screening of the antifungal activity of 500 LAB and PAB strains as fermentates
Garnier et al., 2019 Food Microbiol

32 strains selected
18 Lactobacillus
5 Leuconostoc
9 Propionibacterium

5 Lactobacillus strains selected
with high activity & broad spectrum of action
Tested in combination

→ 2 binary combinations retained to be tested at the pilot scale

Screening results

→ 17% of the milk fermentates inhibited at least 1 fungal target

Antifungal activity: both species- and strain-dependent
Many Lactobacillus strains active

Safety: biogenic amine production and antibiotic resistance

L. harbinensis
L172
L. plantarum
L244
A1
A3
L. rhamnosus
CIRM-BIA1113

Screening & Validation

Conclusions
**Challenge-test**: inoculation of targets

- **50 spores** on the surface
  - *P. commune*
  - *M. racemosus*

- **2 yeast cells/g** in sour cream
  - *R. mucilaginosa*

**Sour cream**

**Cheese**

**Shelf life tests**: 20 min exposition

- 4 weeks incubation at 10° C

- 2 weeks incubation at 10° C

- Ripening
  - 4 weeks at 12° C

**Introduction**

**Conclusions**

**Screening & Validation**

**Antifungal metabolites**
Example of results during scale up (1)

**Sour cream challenge-test against *P. commune***

- **Control**
- **Sorbate**
  - A1: $5 \times 10^6$, $10^6$
  - A3: $2 \times 10^7$, $5 \times 10^6$, $10^6$
  - X1: $10^6$

**Days of total inhibition**

- A1 higher AF activity and spectrum of action compared to A3 and commercial X1
- A1 antifungal activity > *in sour cream* than during screening

*Leyva-Salas et al., 2018 Frontiers in Microbiology*
Example of results during scale up (2)

Shelf life test in semi-hard cheese

A1 antifungal activity > A3 and commercial culture

Leyva-Salas et al., 2018 Frontiers in Microbiology
Analyses of antifungal compounds

screening in two dairy models

two dairy products at the pilot scale

4 dairy products, 2-3 biological replicates
2 antifungal combinations tested at 1-3 inoculation levels

→ 100 samples

Targeted metabolomics

to identify the antifungal metabolites involved in the observed activity
Four methods combined to quantify antifungal compounds:

- **HPLC-UV-R**
- **LC – QToF MS**
- **GC - FID**
- **Headspace trap-GC-MS**

### Organic acids
- Lactic acid
- Acetic acid
- Phenyllactic acid

### Fatty acids
- Hydroxydecanoic acid

### Volatile compounds
- Headspace trap-GC-MS

### Proteinaceous compounds
- Cyclic dipeptides

**Antifungal compounds produced by LAB**

**Introduction**

**Screening & Validation**

**Identification of antifungal metabolites**

**Conclusions**
56 antifungal organic acids targeted

34 detected, present at concentrations ranging from > 5 g/kg to < 5 mg/kg

Leyva Salas et al; 2019 Food Chem
Other antifungal compounds

More differences between dairy products than between controls and products with antifungal cultures

→ Compounds involved in the activity?

and ~30 volatile compounds
Compounds produced by antifungal cultures

✓ ANOVA: 33 compounds present at significantly greater amounts in the presence of an antifungal culture compared to the controls

✓ 2 to 16 compounds according to the antifungal culture and the dairy product
Some ‘key’ antifungal compounds
the most correlated with the antifungal activity observed, in 3 or the 4 dairy products

- Acetic acid
- Hydrocinnamic acid
- Phenyllactic acid
- Hydroxyphenyllactic acid
- Diacetyl
- Acetoin
- Pyroglutamic acid

Identification of antifungal metabolites

Introduction
Screening & Validation
Conclusions
Screening & Validation
Conclusions

An efficient approach to develop flexible antifungal cultures with high antifungal activity and broad spectrum

Strategy key points for a robust screening approach:

✓ Screening in dairy matrices
✓ 4 representative and resistant fungal targets
✓ Challenge-test performed = worst case contamination scenario contamination with 50 spores, incubation at 10-12°C
✓ Constraints related to the potential use also considered including safety, sensory and economic constraints (level of inoculation).

→ Best candidates: 2 combinations of 2 lactobacilli strains:

- L. harbinensis L172
- L. plantarum L244
- L. rhamnosus CIRM-BIA1113
Conclusions

Antifungal compounds

✓ 53 antifungal compounds detected, 33 in significantly higher amounts in at least 1 product inoculated with an antifungal culture compared to the controls

✓ No qualitative differences: all present in the fermented dairy products without antifungal cultures

✓ Present at concentrations below their MIC $\rightarrow$ act in synergy

✓ Presence of other (proteinaceous) antifungal compounds not excluded

✓ Interactions with matrix: pH, fat content

Adjunct cultures versus fermentates

✓ Complementarity between both types of bioprotection solutions

On going: exploration of the action mechanisms of some antifungal compounds at the fungal level