Protective cultures in the food industry

Focus on dairy and meat products

DeFENS
13th January 2016
No additives” is the top claim on new products launched globally, and appears on more than 16% of new products in some regions.

“All natural”, for regulatory reasons, is a much more common claim in North America than elsewhere.

**KEY SELLING POINT:**
FOCUS ON “NATURAL” AND “SAFE”

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**Food & drink new product introductions 2009-14, by selected claims, by region**

- **North America**: All Natural Product (18%), No Additives/Preservatives (12%)
- **Latin America**: All Natural Product (6%), No Additives/Preservatives (0%)
- **Asia Pacific**: All Natural Product (6%), No Additives/Preservatives (0%)
- **Europe**: All Natural Product (6%), No Additives/Preservatives (0%)

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*Source: Mintel*
Macro trend: Factory fear - global issue

Product recalls. Allergy scares. Villainous additives. We’re more wary of what we consume than ever before.

- Consumers mistrust the food industry
- Yet they have more information at their fingertips than ever before, via everyday connectivity
- Consumers want foods that are more natural, less processed, made with familiar ingredients
- The focus is on clean labels, greater transparency, and more artisanal values
- 83% of the consumers regularly check the yoghurt label and prefer if it is simple and natural, and above half agree organic is even healthier, but the price is still first basis of choice for most, though
- ½ of all consumers are willing to pay up to 10% more for clean label products

85% of Chinese consumers agree that they are concerned about the safety of their food these days

1/3 of all consumers say that a clean label would make them eat more yoghurt

49% Of UK consumers trust the food industry to provide food that is safe to eat
Control of indigenous bacteria as part of hurdle technology

Hurdle technology is building obstacles for bacteria

- Hurdles enforce protection/preservation of bacteria
  - Protection on individual hurdles
  - Consider combinations
  - To enhance effect
  - Product adjustments
  - Product developments
Control of indigenous bacteria as part of hurdle technology

Enhance the quality of the dairy product and Protect the brand image

Clean label since these strains are considered starter cultures

The products are more resistant to temperature fluctuations during long-distance distribution

Global expenses reduction
Definition of Microbial Food Cultures (MFC)

“MFC are live bacteria, yeasts or moulds used in food production. MFC preparations are formulations, consisting of one or more microbial cultures including unavoidable media components carried over from the fermentation and components, which are necessary for their survival, storage, standardisation and to facilitate their application in the food production process. MFC preparations may contain one or several microbial species.

Starter cultures are MFC preparations used as food ingredients at one or more stages in the food manufacturing process, which develop the desired metabolic activity during the fermentation or ripening process. They contribute to one or multiple unique properties of the fermented food especially in regard to taste, flavour, colour, texture, safety, preservation, nutritional value, wholesomeness and/or health benefits.

The term "fermented" describes the processes of acidification, maturing, ripening, flavouring, and preserving. The metabolic activity of the microorganisms in the preparations is in any case a fermentative event.”
### Examples of MFC applications

<table>
<thead>
<tr>
<th>Desired effect</th>
<th>Group of microorganisms</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taste</td>
<td>Lactic acid bacteria (LAB)</td>
<td>fermented milk, bread, pickles, olives, fermented sausages, wine, beer, vinegar</td>
</tr>
<tr>
<td></td>
<td>acetic acid bacteria</td>
<td></td>
</tr>
<tr>
<td>Aroma</td>
<td>LAB</td>
<td>Same as taste</td>
</tr>
<tr>
<td></td>
<td>Propionibacteria, staphylococci, yeasts, brevibacteria, <em>Arthrobacter</em> spp., <em>Kocuria</em> sp., <em>Zymomonas</em> sp.</td>
<td>Hard-/semi hard cheese</td>
</tr>
<tr>
<td></td>
<td>Moulds</td>
<td>Smear cheese</td>
</tr>
<tr>
<td>Texture/Consistency</td>
<td>LAB, Moulds, staphylococci, brevibacteria, <em>Arthrobacter</em> sp.</td>
<td>Fermented sausages, fish sauce, bread, beer, wine, kefir, soft cheese, fermented sausages, soy sauce</td>
</tr>
<tr>
<td>Colour</td>
<td>LAB, <em>Kocuria</em> sp., staphylococci, brevibacteria</td>
<td>Fermented sausages</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smear cheese</td>
</tr>
</tbody>
</table>
### Examples of MFC applications

<table>
<thead>
<tr>
<th>Desired effect</th>
<th>Group of microorganisms</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shelf life</td>
<td>LAB, yeasts, <em>Zymomonas</em> sp., propionibacteria, acetic acid bacteria</td>
<td>all lactic acid fermented foods, alcoholic drinks, cheese, vinegar</td>
</tr>
<tr>
<td>Safety</td>
<td>LAB, yeasts, <em>Zymomonas</em> sp. Propionibacteria</td>
<td>all lactic acid fermented foods, alcoholic drinks, cheese</td>
</tr>
<tr>
<td>Gas formation</td>
<td>LAB, yeasts, propionibacteria</td>
<td>bread, cheese, fermented milk, beer, sparkling wine, kefir, baked goods, hard-/semi hard cheese</td>
</tr>
<tr>
<td>Nutritional quality</td>
<td>LAB, yeasts</td>
<td>(improved digestibility, degradation of anti-nutritive compounds in) cereals, pulses, vegetables</td>
</tr>
<tr>
<td>Technical aids</td>
<td>LAB, yeasts, moulds</td>
<td>rye-sourdough (bakeability), sour malt (beer), baked goods (gas), soy sauce (enzyme source)</td>
</tr>
</tbody>
</table>
Continuum between the different types of cultures

- Starter Cultures
- Protective Cultures
- Probiotic Cultures

Cultures species with a long history of safe use.
Protective cultures definition:

*Deliberate application of microorganisms to control the bacteriological status in a product without changing the technological and sensory quality of the product considerably*

- Not a preservative
- No promised extension of shelf-life
- No E-number (yet)
Legal situation of protective cultures - EU

- Only active cultures that give extra consumer safety (i.e., protects against pathogens) should be called “protective” as generally for fermented food the normal starter culture are used (through its acid production…) to enhance shelf-life, but naturally also may give change in flavour, texture, nutritional value etc.

- Inactive cultures, sold only because of the contained metabolites to prolong shelf-life, are, legally seen, not cultures but are additive and principally must be labelled as such with E-number…

- Protective cultures for not traditionally fermented products, like cooked meat, fish, etc., may not (officially) prolong or extend shelf-life but only inhibit pathogens and should be labelled

- In other jurisdictions, like as example USA, “fermented milk” and “fermented sugar”, so principally just the metabolites or in-active cultures, can be added and labelled as such, hence possible to add high amounts of acids and bacteriocins for their anti-microbial effect
EU legislation concerning *L. monocytogenes*

- Regulation EU No. 2073/2005 (updated No. 1441/2007) with focus on consumer **protection**
  - Minimise waste of analyses/money with not useful information
- Ready-to-eat food
  - <100 CFU *Listeria monocytogenes*/g at the end of shelf-life
- Total cell count/plate count/aerobic microorganisms
  - Change in number may indicate change in hygiene
  - No other useful information
  - Only demanded on minced meat to determine shelf-life

- Special local legislation possible
Protective culture dairy applications*

- Raw milk «protection» (LRB)

- High pH fresh cheese «protection» against pathogens (LRB o.o.)

- Cheese and fermented milk «protection» against Yeast & (foreign) moulds (LRB, LPRA, CNB a.o.)

- Cheese «protection» against Listeria m. (LPAL, CNB AL a.o.)

- Cheese «protection» against late blowing (LC 4P a.o.)

- Cheese «protection» against biogenic amines (LC 4P a.o.)

*examples/references from praxis can unfortunately not be given as the clients generally do not want us to tell about what they use
Protective cultures other applications

- As part of starter cultures for meat fermentation to inhibit *Listeria* (Pc. Lb.s. a.o.)
- On raw, smoked and smoked meat products to inhibit *Listeria* (CNB)
- On raw, graved and smoked fish to inhibit *Listeria* (CNB)
- On cooked potatoes to inhibit growth of *E. coli* + Ps.a. (DY)
- In salads to inhibit *Listeria, Salmonella* etc. (Lc.i., CNB etc.)
Contamination Risk

**Endogenous Microbiota**
(raw material...)

**Environmental Microbiota**
(tools, equipment..)

**Environmental Microbiota**
(operator..)

**Transport, Storage, Consumption**
Level of bacteria in food

- Food may contain three categories of bacteria
  - The bad
    - Pathogenic bacteria
  - The ugly
    - Spoilage bacteria
  - The good
    - Harmless bacteria/starter cultures

Good Manufacturing Practise (GMP)
- Minimise level of pathogenic bacteria – authorities and manufacturers
  - Sick people are expensive
- Minimise spoilage bacteria – manufacturers
  - Shelf-life
  - Returned goods
How to evaluate bacteria in food

- Safety
  - Foodborne illness
- Pathogenic
  - Total cell count
- Harmless
  - Good
- Spoilage
- Shelf-life
- Maybe production hygiene
Protective Lactic Acid Bacteria (LAB)

General features:
- Being able to grow at storage temperature
- Limited sensory impact
- No/limited influence on pH
- **No preservative** and possible influence on shelf-life depends on which bacteria are present in the matrix

Two protective systems are useful in meat products:
- **Competitive exclusion**
  - Compete with the indigenous biota on:
    - Easily fermentable nutrients
    - Rest oxygen in vacuum-packed and MAP
- **Production of bacteriocins & other compounds**
  - Peptides with specific mode-of-action
- (Combination of the two systems)
Bacterial competition

Culture not able to grow with (at least) the same speed as indigenous biota:

- Indigenous bacteria will over-grow not "active" culture
- No advantage of added culture

Growth depends on
- Meat matrix
- Production and storage temperatures
- Packaging – vacuum or MAP, which minimise growth of Gram-negative bacteria
The level of bacteria is the same after a period in meat products with or without culture added.

- Competitive culture gives an uniform and controlled production.
Microbial diversity

- Without culture added
- With protective culture added
Fermentation products: organic acids

- Lactic acid
- Acetic acid
- Propionic acid
- Phenyllactic acid
- Butyric acid
- Valeric acid
- Caproic acid
Fermentation products: other end products

- Hydrogen peroxide
- Diacetyl
Proteinaceous compounds

Diverse group of antimicrobial peptides

Common features:
- hydrophobic and hydrophilic end;
- 20-50 a.a. in length;
- cationic properties.

Highly hydrophobic antifungal peptide from *L. coryniformis* and *L. amylovorus*: increased production when ethanol, formic or acetic acids are present.
Low molecular weight compounds

Reuterin
(3-hydroxypropionaldehyde)
Produced from glycerol by starving cells under anaerobic conditions

Diketopiperazines

Cyclo (L-Phe-L-Pro)
Cyclo (L-Leu-L-Pro)
Cyclo (Gly-Leu)
### Examples of antifungal LAB
Magnussen 2003

<table>
<thead>
<tr>
<th>LAB isolate*</th>
<th>Activity spectrum</th>
<th>Compounds</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. plantarum</em></td>
<td>Saccharomyces cerevisiae</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em> subsp. <em>rhamnosus</em> LC-705</td>
<td>Candida lusitaniae Aspergillus niger Fusarium spp. Penicillium spp.</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND = Not determined

*Some species have through taxonomic revisions received new species identities, which are not taken into account here.*
**Examples of antifungal LAB**

Magnussen 2003

<table>
<thead>
<tr>
<th>LAB isolate*</th>
<th>Activity spectrum</th>
<th>Compounds</th>
<th>Reference</th>
</tr>
</thead>
</table>

1 Sodium acetate from the MRS substrate was involved in the inhibitory action of lactic acid bacteria towards several moulds: the additional effect of other compounds was not determined.
Bacteriocin producing protective cultures

Table 1 | Suggested classification scheme for bacteriocins

<table>
<thead>
<tr>
<th>Classification</th>
<th>Remarks/suggestions</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanthionine-containing bacteriocins/lantibiotics</td>
<td>Includes both single- and two-peptide lantibiotics; up to 11 subclasses have been proposed(^9)</td>
<td>Single-peptide: nisin, mersacidin, lacticin 481; two-peptide: lacticin 3147, cytolsin</td>
</tr>
<tr>
<td><strong>Class II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-lanthionine-containing bacteriocins</td>
<td>Heterogeneous class of small peptides; includes pediocin-like (subclass a bacteriocins), two-peptide (subclass b bacteriocins), cyclic (subclass c; formerly class V), non-pediocin single linear peptides (subclass d)</td>
<td>Class IIa: pediocin PA1, leucocin A; class IIb: lactacin F; class IIc: enterocin AS48, reuterin 6; class IIId: lactococcin A, dierugin A</td>
</tr>
<tr>
<td><strong>Bacteriolysins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-bacteriocin lytic proteins(^\d)</td>
<td>Large, heat-labile proteins, often murein hydrolases</td>
<td>Lysostaphin, enterolysin A</td>
</tr>
</tbody>
</table>

* Class IV bacteriocins (bacteriocins with non-proteinaceous moieties) are not included as no members have been demonstrated.

\(^\d\) Suggested that these are no longer considered bacteriocins (see main text).
Bacteriocin based «protection»
Raw milk hygienic issues

- Countries with hygiene issues, many small farms and too high storage temperature
- But also with good cooling, if the amount of psychrotrophic bacteria becomes too high, issues with the quality of the milk regarding flavour as well as stability in UHT because of high enzymatic activity still after heat treatment and/or because of extra heat resistant spore formers and maybe also with yield in cheese production
- Even if they all should be killed by pasteurization high counts of potential pathogen, Enterobacteriaceae and *E.coli* etc. are not wanted

➢ Use of a protective strain/s, does not give negative flavour/texture impact in the final dairy products, does not inhibit the normally used starter cultures, but still has a strong inhibitory effect on the unwanted microorganisms
Psychrotrophic bacteria:

- aerobic
- Able to grow at low temperature
- Thermosensitive (64 °C 20 s)

About 10% of the psychrotrophic bacteria in the milk are **pseudomonas**. (Problem: count > 10,000 CFU)

• Able to produce exocellular and thermoresistent enzymes!!
  
  • Lypases
  • Protease

Source: ENIL BIO - 2007
Raw milk «protection»
ex 1. LR B - 5x10E4/ml

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>SILO CONTROL</th>
<th>SILO c/ LRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4,6</td>
<td>5,7</td>
</tr>
<tr>
<td>24</td>
<td>6,7</td>
<td>1,7</td>
</tr>
<tr>
<td>48</td>
<td>4,4</td>
<td>1,7</td>
</tr>
</tbody>
</table>

Time of action LR B
min 7-8 h
### Raw milk «protection»

**ex 1. LR B - 5x10E4/ml**

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>PSYCHROTROPHIC BACTERIA x 10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SILO CONTROL</td>
</tr>
<tr>
<td>0</td>
<td>3,9</td>
</tr>
<tr>
<td>24</td>
<td>5,8</td>
</tr>
<tr>
<td>48</td>
<td>3,1</td>
</tr>
</tbody>
</table>

**Time of action LR B min 7-8 h**
Raw milk «protection»
ex 2. LR B - 5x10E4/ml

<table>
<thead>
<tr>
<th>Fecha</th>
<th>Tiempo de conservación (hrs.)</th>
<th>Mesófilos x 10^4</th>
<th>Psicroтроfos x 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tk Control</td>
<td>Tk c/cultivo</td>
<td>Tk Control</td>
</tr>
<tr>
<td>07/04/2014</td>
<td>0</td>
<td>250</td>
<td>270</td>
</tr>
<tr>
<td>08/04/2014</td>
<td>24</td>
<td>310</td>
<td>200</td>
</tr>
<tr>
<td>09/04/2014</td>
<td>48</td>
<td>370</td>
<td>210</td>
</tr>
<tr>
<td>10/04/2014</td>
<td>72</td>
<td>580</td>
<td>370</td>
</tr>
</tbody>
</table>

Time of action LR B 72 h
## Raw Cream «protection»

<table>
<thead>
<tr>
<th>Test</th>
<th>Raw cream without LR B</th>
<th>Raw cream with LR B</th>
<th>AEROBIC MESOPHILIC BACTERIA</th>
<th>PSYCHROTROPHIC BACTERIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1</td>
<td></td>
<td></td>
<td>44.000</td>
<td>3.000</td>
</tr>
<tr>
<td></td>
<td>Raw cream with LR B</td>
<td></td>
<td>6.100</td>
<td>100</td>
</tr>
<tr>
<td>Test 2</td>
<td></td>
<td></td>
<td>233.000</td>
<td>124.000</td>
</tr>
<tr>
<td></td>
<td>Raw cream with LR B</td>
<td></td>
<td>222.000</td>
<td>79.000</td>
</tr>
<tr>
<td>Test 3</td>
<td></td>
<td></td>
<td>920.000</td>
<td>8.800</td>
</tr>
<tr>
<td></td>
<td>Raw cream with LR B</td>
<td></td>
<td>180.000</td>
<td>8.000</td>
</tr>
</tbody>
</table>
Fresh and soft not matured and weakly fermented cheese types with high pH (from above 6 and down to 5) have issues with out growth of unwanted microorganism

Some pathogens such as Gram⁻ like Enterobact. incl. Salmonella and E.coli, Pseudomonas as well as Gram⁺ like Staph., Bacillus, Clostridia, Listeria etc. as well as yeast and moulds)
Example 1
4 weeks at 8°C
Example 1
4 weeks at 14°C
Fresh cheese «protection»
discussion of results

- *Lactobacillus* (LRH) and/or *Carnobacterium* can give good inhibition of unwanted growth contaminants
- When the cold chain is broken the effect becomes higher as the protective cultures grow more
- Depending on strain (lactose +/-) the effect is more but then also gives stronger pH drop that depending on product may also influence the quality/flavour significantly when stored too warm
- Applied so far for different fresh cheese types (Sfatid, Cottage Cheese, soft Mozzarella a.o.) in Italy, Israel a.o. countries
LAB «protection» against yeast & moulds

LAB unfortunately have limited effect against Y & M

Two *L. rhamnosus* and a *L. plantarum* have reasonable good effect and not found to promote the growth of yeast strains

For yeast the gas and yeasty flavour formation is more inhibited than the counts, hence a small inhibition and delay in growth can still give a good effect in prolongation of shelf

Mode of action not really know!
LAB «protection» against yeast, example
Evaluation of antifungal activity in LAB vitro by the overlay agar milk assay

Penicillium:
a mixture of *P. roqueforti* strain N and strain A

Yeast:
a mixture 1:1:1 of *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae*.

The overlay consisted of 200 µl of potato dextrose (PDA) soft agar (0.5 g/l) containing: 10^4 or 10^3 or 10^2 spores or cells/ml.

Plates were incubated at 30°C

### Microrganisms used

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Internal code</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penicillium roqueforti</em></td>
<td>PRN + PRA</td>
</tr>
<tr>
<td><em>Kluyveromyces marxianus</em></td>
<td>KLM02</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>SCH03</td>
</tr>
<tr>
<td><em>Debaryomyces hansenii</em></td>
<td>DH01</td>
</tr>
</tbody>
</table>
LAB «protection» against moulds

PR on yoghurt inhibited by different strains

+ *Penicillium*

*Penicillium* inoculated $10^5$ spore/ml

Yeast (blend of DH, KLM, SC)$10^2$ CFU/ml

After 10 days of incubation at 10 °C

The best *Penicillium* sporulation-inhibitor are: LRH08, LA03, **LRH02, LPC17, LF07**.

Weak inhibition for PBF02, LC10
# Anti Y&M screening

10^4 CFU/ml yeast + 10^4 CFU/ml mould

Incubation: 6 days

<table>
<thead>
<tr>
<th>Strain</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Lb. plantarum BG 112</td>
<td></td>
</tr>
<tr>
<td>3 Lb. plantarum LP AL</td>
<td></td>
</tr>
<tr>
<td>4 Lb. paracasei BGP1</td>
<td></td>
</tr>
<tr>
<td>6 Lb. casei LPC 4 P1</td>
<td></td>
</tr>
<tr>
<td>7 C. divergens CNB AL</td>
<td></td>
</tr>
<tr>
<td>8 Lb. rhamnosus LR B</td>
<td></td>
</tr>
<tr>
<td>10 Lb. acidophilus LA03</td>
<td></td>
</tr>
<tr>
<td>11 Lb. brevis LBR12</td>
<td></td>
</tr>
<tr>
<td>12 B. animalis lactis BLC01</td>
<td></td>
</tr>
<tr>
<td>13 P. freudenreichii PB01</td>
<td></td>
</tr>
<tr>
<td>18 Lb. paracasei HOLDBAC YM-C</td>
<td></td>
</tr>
<tr>
<td>19 P. freudenreichii HOLDBAC YM-C</td>
<td></td>
</tr>
<tr>
<td>22 Lb. fermentum LF07</td>
<td></td>
</tr>
</tbody>
</table>

- **Lb. plantarum** BG112
- **Lb. plantarum** LP AL
- **C. divergens** CNB AL
- **Lb. rhamnosus** LR B
- **Lb. brevis** LBR12
- **B. animalis lactis** BLC01
- **P. freudenreichii** PB01
- **Lb. paracasei** HOLDBAC YM-C
- **P. freudenreichii** HOLDBAC YM-C
- **Lb. fermentum** LF07
- **HOLDBAC YM-C**
Example of anti-mould activity on model cheese
Example of cheese inoculated with LRB
1. Prepare Listeria agar, cool at ~50°C and add cells of Listeria to reach a final concentration of ~10^6 CFU/ml

2. Pour 16ml into Petri dish

3. Cut 4 holes* (keeping an equal distance between each hole), using a sterile size 4 cork borer

4. Seal the bottom of each well by adding 50 µl of agar-agar. Allow solidification of the bottom layer.

5. Add 50µl of the test strain supernatant into each well.

5. After incubation, determine the inhibition (by measuring the halo size/recording if there is a clear cut or a fading inhibition)

Halo size
Bacteriocin producers against Listeria, Clostridia etc.
Anti-Listeria activity in Gorgonzola

Example 1

*L. plantarum – L. rhamnosus – E. faecium*

- Control
- Cheeses treated with $10^6$ CFU cm$^{-2}$ on surface by sprying
- Three treatments (5, 20, and 40 days)
- Samples collected during ripening
Anti-Listeria activity in Gorgonzola

Example 1

- Control: 32% of cheese surface contaminated by *Listeria* (25g), 10% also positive in 1g!

- Treated: 6% of cheese surface contaminated by *Listeria* (25g), 0 positive in 1g!
Anti-Listeria activity in Gorgonzola

Example 2

Cheese surface treated with *Carnobacterium* salt solution

⇒ Also effective against surface foreign moulds growth
Cheese «Protection» against Clostridia

- Nitrates addition
- Lysozyme addition
- Use of a strain producer of a bacteriocin active against Clostridia
Bac<sup>+</sup> producers in a mini-cheese

Gouda cheese production in laboratory scale

1. Inoculum: 30 min
   - CaCl<sub>2</sub>
   - Starters
   - Cl. tyrobutyricum spores
   - +/- bacteriocin producer strain/s

2. Renneting and curd cutting: 45 min
Bac⁺ producers in a mini-cheese

3. Stirring and whey off (30%): 30 min

- Stirring in vat
- Low speed centrifugation

4. Stirring, heating and hot water addition: 40 min

5. Whey off: 60 min

- Pressing systems
- Low speed centrifugation

6. Pressing: 30 min
Bac⁺ producers in a mini-cheese

7. Brining: 0 (to simulate cheese core)

8. Vacuum pack

10Kg vs. 15g

9. Ripening

~11-18°C

~20°C (to be sure!)
Bac⁺ producers in a mini-cheese

Sampling and cheese analysis:

- samples collected after 4 and 8 weeks of ripening;
- free fatty acid extraction (FFA) and Gas Chromatography analysis for the detection and the quantification of the butyric acid;
- well diffusion assay of small portion of cheese;
Bac⁺ producers in a mini-cheese

Mini-cheese added with single bacteriocin producer *Lactococcus lactis* strain:

C) + DBC32 (*subsp. lactis* isolated from wastewater, Nisin Z producer)
D) + SL28 (Nisin A)
E) + SD66 (Nisin A)
F) + SL147 (Lacticin 481 and Nisin Z)
G) + U6_3 (lacticin 3147)
H) + N245 (*subsp. cremoris*, isolated from grass, producer of unknown molecules)
A) NOT added Bacteriocin producer (considered as 100%)
Bac⁺ producers in a mini-cheese

Mini-cheese added of COUPLE of bacteriocin producers:

- **I)** + U6_3 and SL147 (Lacticin 3147 and Lacticin 481 producers)
- **L)** + SL28 and U6_3 (Nisin A and Lacticin 3147)
- **M)** + SL28 and SL147 (Nisin A and Lacticin 481)
- **N)** + U6_3 and SD66 (Lacticin 3147 and Nisin A)
- **O)** + SL28 and DBC32 (Nisin A and Nisin Z)
- **A)** NOT added Bacteriocin producer (considered as 100%)

![Graph showing relative amount of butyric acid after 1 and 2 months of ripening in different samples.](image)
Cheese «Protection» against Clostridia

Range of culture solutions that can be used to inhibit Clostridia and other nisin (as well as lactococcin and thermophilin) sensitive gas-forming MO that may course spoilage in soft, semi-soft, semi-hard and hard cheeses, even for cheese made from milk of silage fed cows without nitrate/Lysozyme.

Depending on cheese type, wanted flavour and technology this is not always the optimal solution or not always giving effect enough against Clostridia or decarboxylating LAB (producing biogenic amines), of which reason we also selected as well as have licenced strains of NSLAB from KU Science that also can inhibit Clostridia and other late blowing microorganism.
NSLAB used for Cheese «Protection» against Clostridia
NSLAB used for Cheese «Protection» against late blowing
NSLAB used for Cheese «Protection» against late blowing
NSLAB used for Cheese «Protection» against late blowing
Main spoilage bacteria in meat products

- Gram-negative:
  - Need oxygen to grow
  - Relative sensitive to pH decline

Advantages of applying LAB

- Microaerophilic
  - Grow in vacuum-packed and MAP products
- Part of hurdle technology – control
- Competitive exclusion inhibits e.g. *Listeria*, *Brochothrix* and spoilage bacteria such as gas producing LAB
- Bacteriocin production inhibits/kills *Listeria monocytogenes*
- Controlling quality and safety
Mode of action: competitive exclusion

- Characteristics of protective *Lactobacillus sakei*:
  - Grows down to 2°C
  - For red and white meats
  - Competitive in vacuum-packed and MAP products
  - Microaerophilic
  - Limited acidification
  - Limited proteolytic and lipolytic activity
  - Impact on shelf-life depends on factors determining shelf-life
Mode of action: bacteriocin production

- *Carnobacterium* producing bacteriocin at low temperatures
  - Grow down to 2°C
  - Useful in all meat and fish applications
  - Not a strong competitor
  - Only for enhanced safety
  - No acidification
  - No sensory impact

- On license from French University
  - Documentation available
    - Efficient against 57 *L. monocytogenes* strains
Protective 1: BOM-13, BOX-74, FP-18

- Meat without nitrite applied or cooked products packed in vacuum or MAP
- Lyocarni BOM-13 with *Lb. sakei*
- Lyocarni BOX-74 with *Lb. sakei* and *Carnobacterium*
- Lyoflora FP-18 with *Carnobacterium*

- Both strains grow at low temperatures
- Competitive exclusion by *Lb. sakei*
  - Inhibit primarily spoilage bacteria
- Bacteriocin production by *Carnobacterium*
  - Inhibit/kill *L. monocytogenes*
Protective 2: BXH-69, BMX-37

- Fresh meat products with nitrite applied packed in vacuum or MAP
  - Lyocarni BXH-69 with *Lb. sakei*
  - Lyocarni BMX-37 with *Lb. sakei* and *Carnobacterium*
  - Both with added Staphylococci

- Has in addition to protection
  - Enhanced colour formation
  - Enhanced colour stability
Concentration of protective culture

- Apply $10^5$–$10^7$ CFU/g or cm$^2$ meat depending on initial bacterial load. Recommendable >$5 \times 10^5$ CFU/g

- Protective strains will grow when determining aerobic total cell count

- It is necessary to have at least 10 times more “good” bacteria to control the indigenous bacteria with minimum $10^5$ CFU/g
**Application of protective culture**

<table>
<thead>
<tr>
<th>Type of meat</th>
<th>How to apply</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh minced meat</td>
<td>Sprinkled into the mince</td>
</tr>
<tr>
<td>- Whole pieces</td>
<td>Spray (or dip) on surface</td>
</tr>
<tr>
<td>- Injected brine</td>
<td>Dissolve in brine</td>
</tr>
<tr>
<td>Frozen meat</td>
<td>Apply before freezing and it will work when the product is thawed</td>
</tr>
<tr>
<td>Processed meat</td>
<td>Spray (or dip) it on surface after heat treatment and cooling</td>
</tr>
</tbody>
</table>
Challenge test 1: cooked ham

- Smoked, cooked, sliced ham
- Lyocarni BOX-74
  - Sprayed on during slicing
- MAP
- Stored at 8°C
- Control initially
  - 1.7x10^3 CFU aerobic bacteria/g
  - No L. monocytogenes detected in 25 g
Challenge test 2: cold-smoked filet

- Cured, cold-smoked, sliced filet
  - Lyocarni BOX-74
    - Sprayed on during slicing
  - MAP
  - Stored at 5°C
  - Control initially
    - <10^4 CFU aerobic bacteria/g
    - No *L. monocytogenes* detected in 25 g
Challenge test 3: emulsion sausages 1

Cooked emulsion sausages
- Lyocarni BOX-74
  - Cooling and removal of skin
  - Sprayed on before packaging
- MAP
- Stored at 8°C
- Control initially
  - $3.3 \times 10^3$ CFU aerobic bacteria/g
  - No <i>L. monocytogenes</i> detected in 25 g
Challenge test 3: emulsion sausages 2

1. Sausages with BOX-74 and Listeria applied

2. Sausages with Listeria applied
Fish matrix

Fish different from other types of meat

- pH
  - Higher, easier growth of bacteria – also *Listeria monocytogenes*

- Protein structure
  - Different structure – other types of starter cultures needed

- Biota
  - Different microorganisms growing
LAB growth in smoked salmon

![Graph showing LAB growth in smoked salmon with CFU/g on the y-axis and Time/Days on the x-axis. Two lines are shown: Av. control/5°C and Av. FP-18/5°C.]
pH in smoked salmon

![pH Graph]

- **Control/5°C**
- **FP-18/5°C**
Challenge test: smoked salmon at 5°C
Application examples (aiming at $>10^5$ CFU/g)

- **Injection**: with 3.5% injected weight gain; 3.5 kg brine with $5.2\times10^{10}$ CFU Lyoflora FP-18 should be applied per 100 kg fish

- **Spraying (or dipping) on dried surface**: disperse 5 g culture in 1 litre cold water and lightly moisture the surface of 100 kg fish with the solution

- **Dry salting**: disperse 5 g culture in 1 litre cold water. 1 ml solution should be applied per 100 g surface dried fish after normal salting and desalting process but before smoking
Pros and cons for protective cultures

Pros

- Enhanced quality - inhibition of spoilage bacteria
- Enhanced safety - inhibition of pathogenic bacteria
- Biological system
- Stable sensory quality – but different from control during storage
- Might extend shelf-life depending on initial spoilage bacteria and factors setting shelf-life

Cons

- Relatively high initial cell count compared to untreated product
- Application: extra production step for some products
- Unacceptable/changed sensory quality
- Unacceptable pH decline
Thank You for Your attention
Any questions?

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