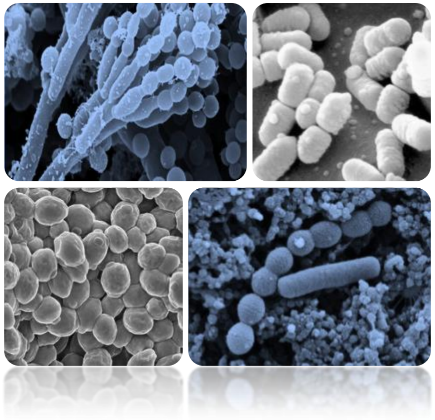
Protective cultures in the food industry



Focus on dairy and meat products

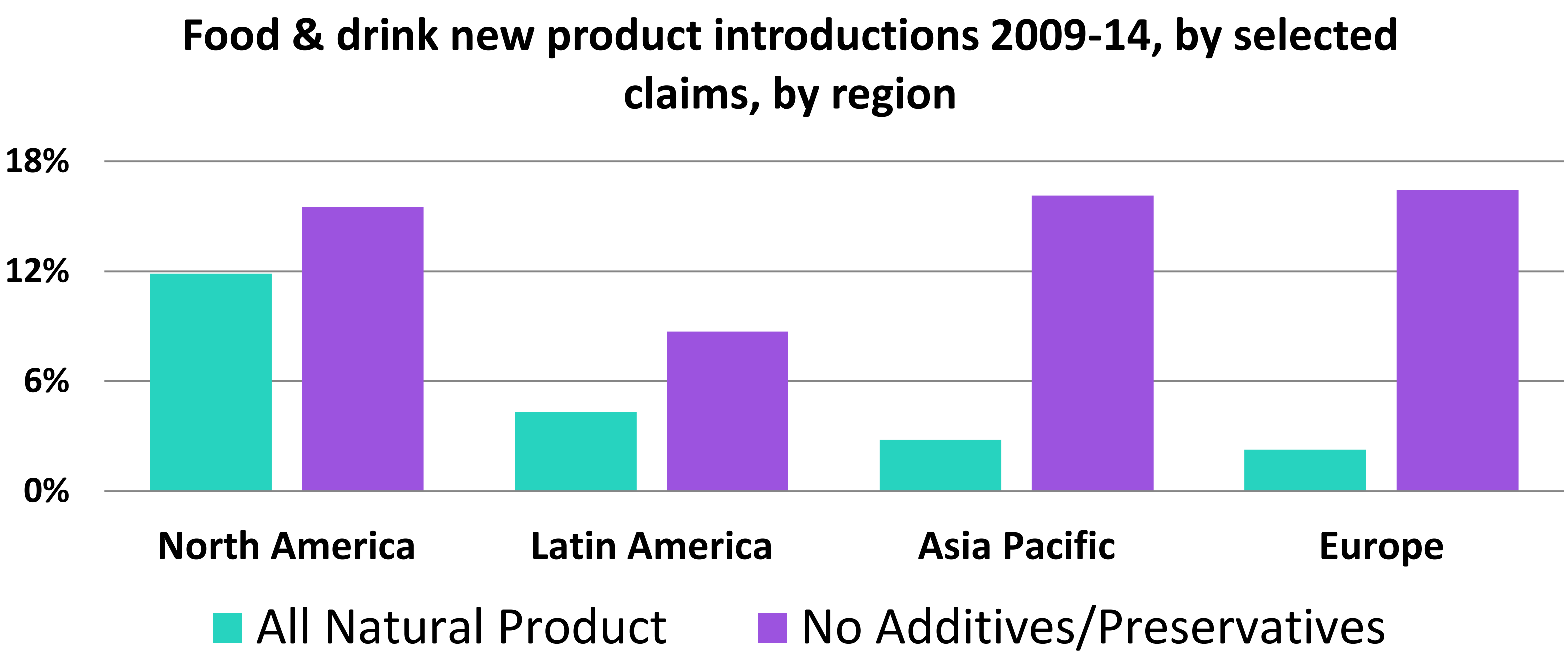
DeFENS
13th January 2016

KEY SELLING POINT :

FOCUS ON "NATURAL" AND "SAFE

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



- Source: Mintel

Macro trend:
Factory fear- global issue

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

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

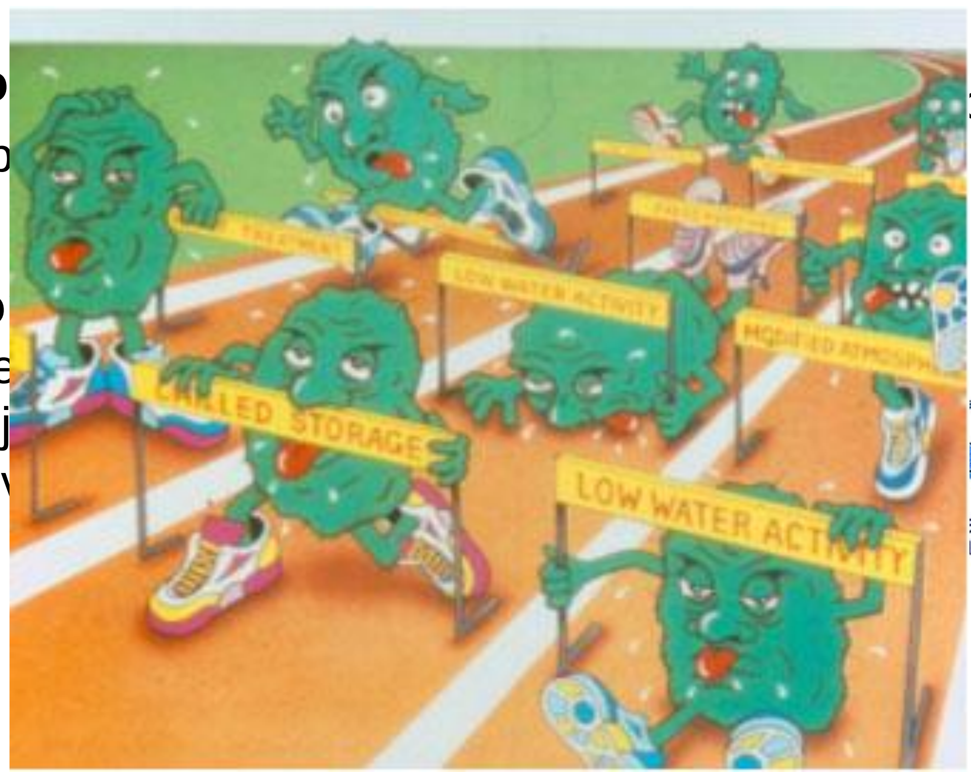
KEY SELLING POINT : CONTROL

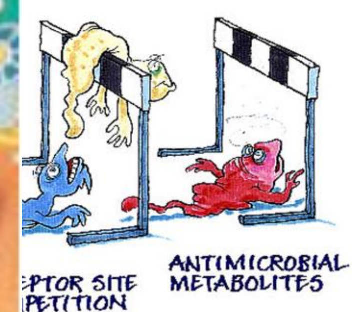
Control of indigenous bacteria as part of hurdle technology

Hurdle technology is building obstacles for bacteria

- **Hurdles enfo** cteria
  - Protection/p

- **Consider co**
  - To enhance
  - Product adj
  - Product dev





KEY SELLING POINT :

CONTROL FROM SPOILING

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.”

EFFCA European Food & Feed Cultures Association

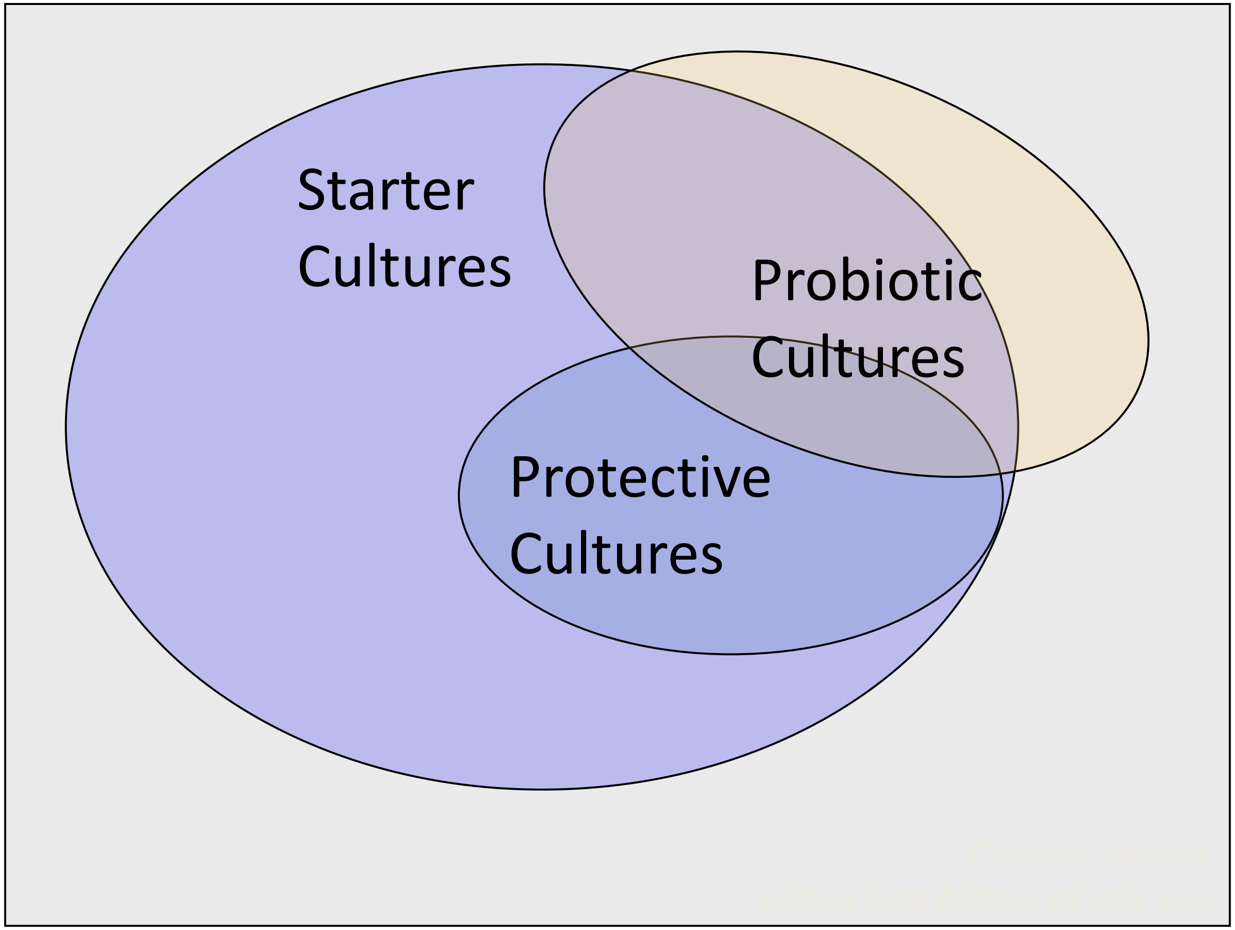
Examples of MFC applications

Desired effect	Group of microorganisms	Food
Taste	Lactic acid bacteria (LAB) acetic acid bacteria	fermented milk, bread, pickles, olives, fermented sausages, wine, beer, vinegar
Aroma	LAB Propionibacteria, staphylococci, yeasts, brevibacteria, *Arthrobacter* spp., *Kocuria* sp., *Zymomonas* sp. Moulds	Same as taste Hard-/semi hard cheese Smear cheese Fermented sausages, fish sauce, bread, beer, wine, kefir, soft cheese, fermented sausages, soy sauce
Texture/Consistency	LAB, Moulds, staphylococci, brevibacteria, *Arthrobacter* sp.	fermented milk, pickles, bread, fermented sausages, cheese
Colour	LAB, *Kocuria* sp., staphylococci, brevibacteria	Fermented sausages Smear cheese

Examples of MFC applications

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

Continuum between the different types of cultures



Protective cultures

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)

EFFCA
European Food & Feed Cultures Association

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*

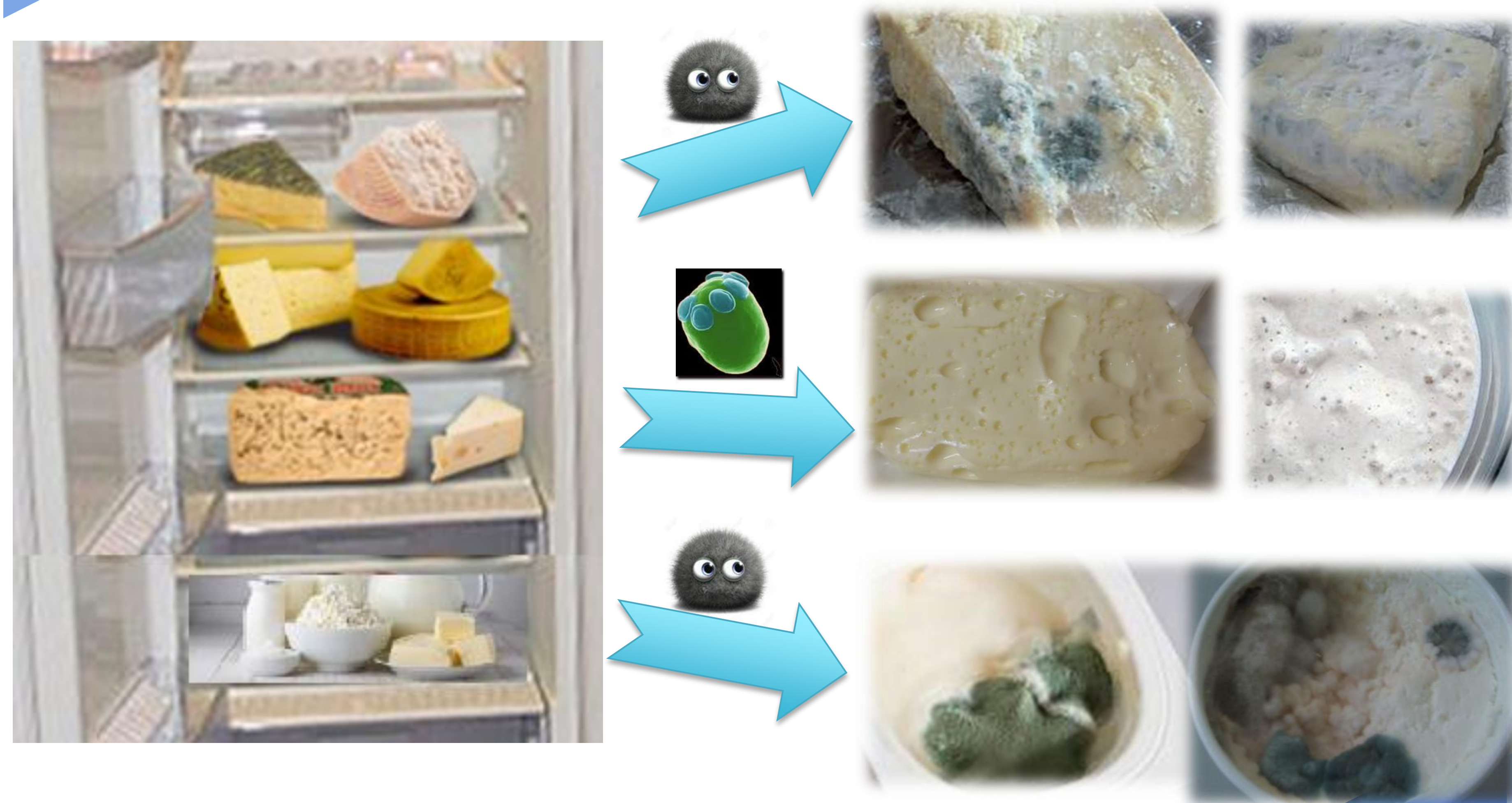
- Raw milk «protection» (LRB)
- High pH fresh cheese «protection» against pathogens (LRB o.o.)
- Cheese and fermented milk «protection» against Yeast & (foreign) moulds (LRB, LPRA, CNB a.o.)
- Cheese «protection» against Listeria m. (LPAL, CNB AL a.o.)
- Cheese «protection» against late blowing (LC 4P 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, smooked 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.l., CNB etc.)

SPOILAGE RISK



Contamination Risk

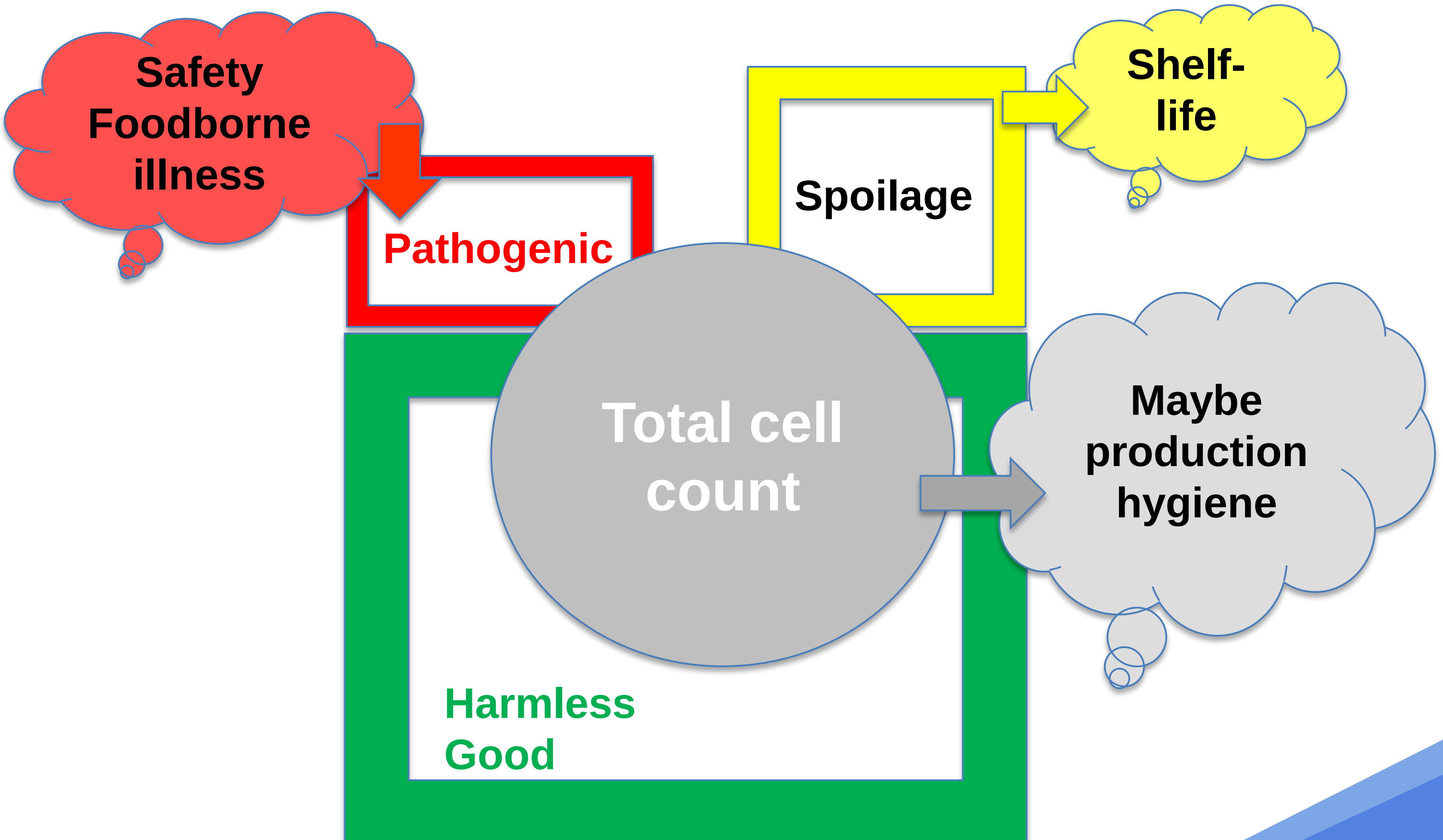
Level of bacteria in food

- 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

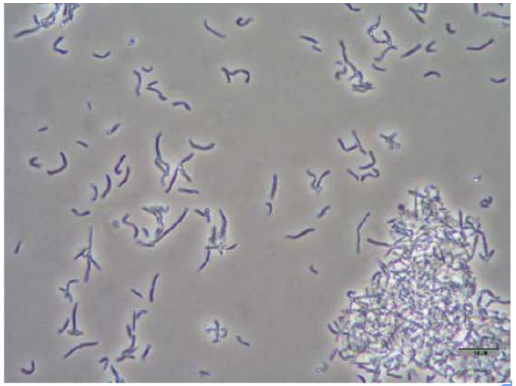
Total cell count

- How to evaluate bacteria in food



Protective Lactic Acid Bacteria (LAB)

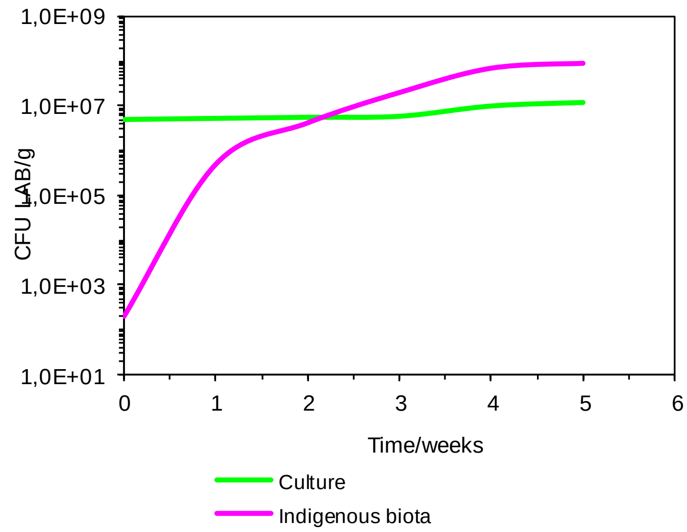
- General features:
  - Being able to grow at storage temperature
  - Limited sensory impact
  - No/limited influence on pH
  - **No preservative** and possible influence on shelf-life depends on which bacteria are present in the matrix
- Two protective systems are useful in meat products:
  - **Competitive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



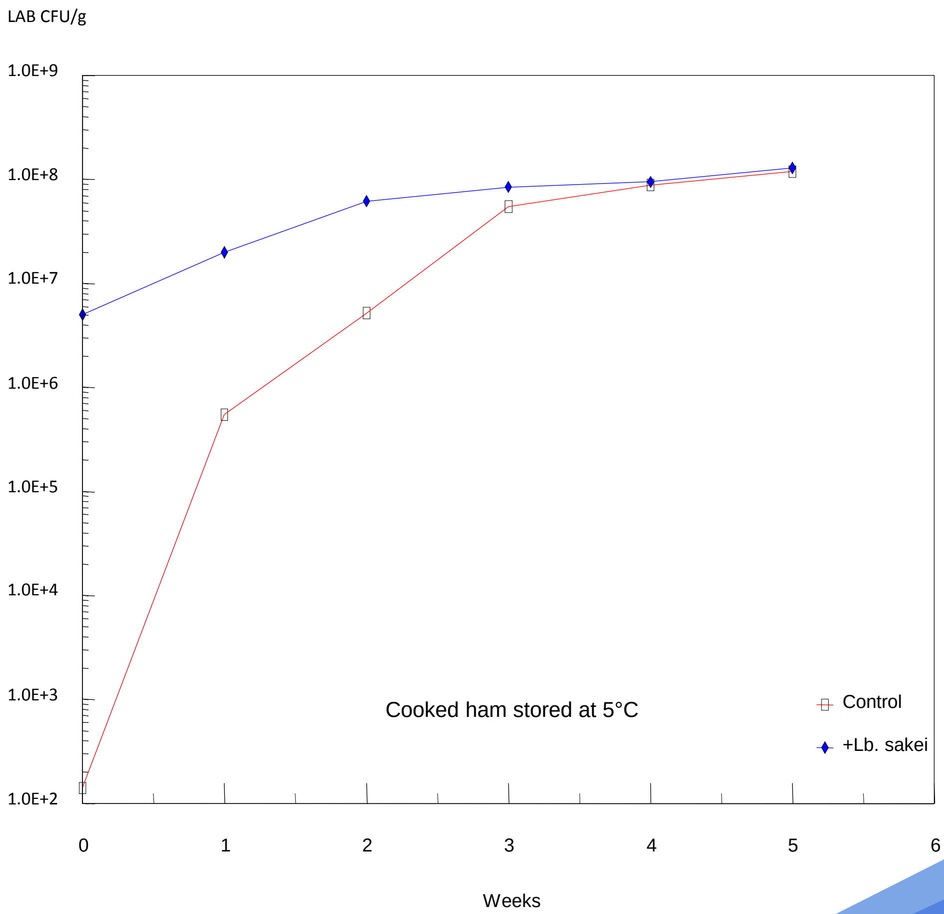
Growth depends on

- Meat matrix
- Production and storage temperatures
- Packaging – vacuum or MAP, which minimise growth of Gram-negative bacteria

Controlled biota

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

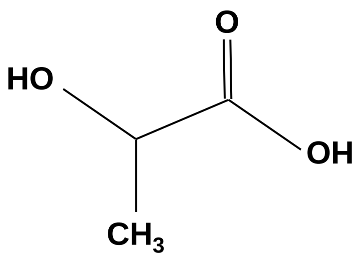


Microbial diversity

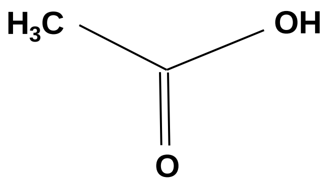
- Without culture added

- With protective culture added

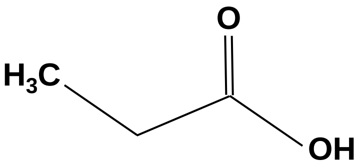
Fermentation products: organic acids



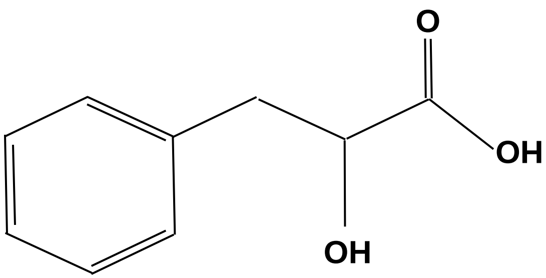
Lactic acid



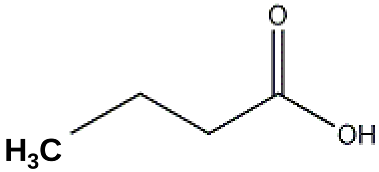
Acetic acid



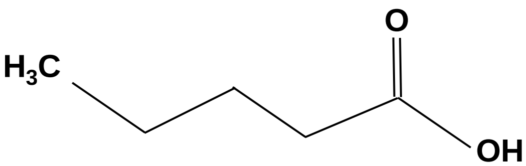
Propionic acid



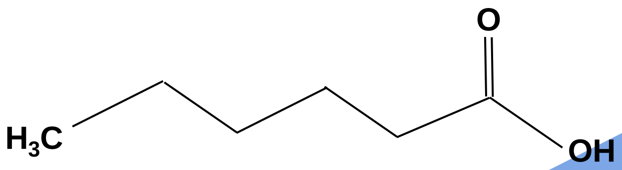
Phenyllactic acid



Butyric acid

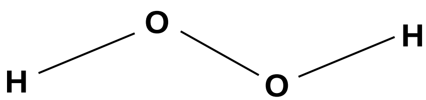


Valeric acid

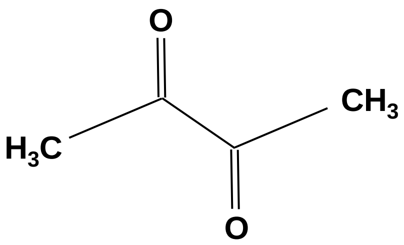


Caproic acid

Fermentation products: other end products



Hydrogen peroxide



Diacetyl

Proteinaceous compounds

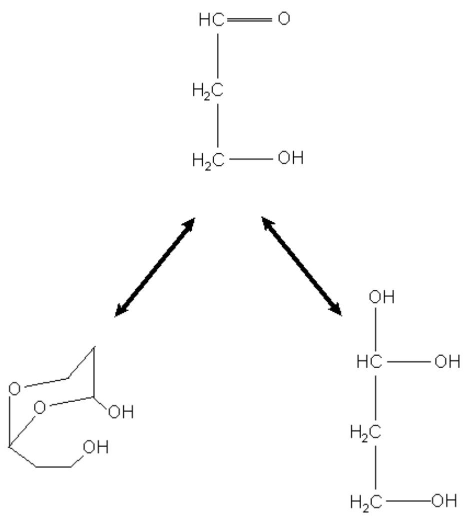
Diverse group of antimicrobial peptides

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

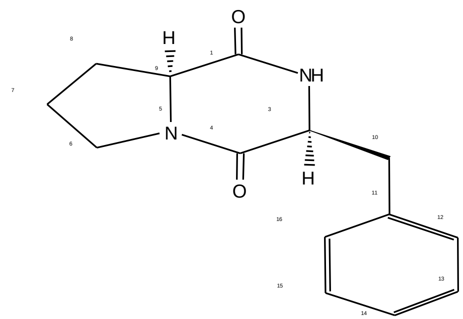


Reuterin

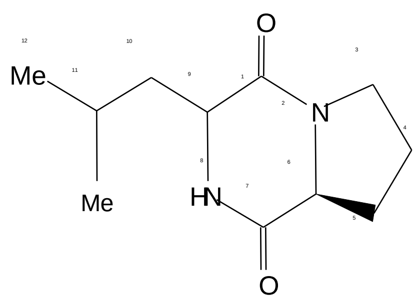
(3-hydroxypropionaldehyde)

Produced from glycerol by starving cells under anaerobic conditions

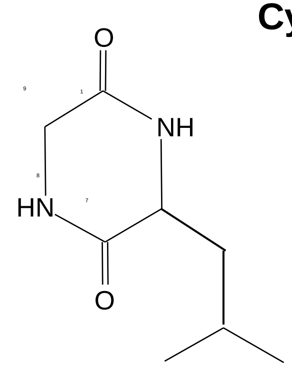
Diketopiperazines



Cyclo ($_L$Phe-$_L$Pro)



Cyclo ($_L$Leu-$_L$Pro)



Cyclo (Gly-Leu)

Examples of antifungal LAB

Magnussen 2003

LAB isolate*	Activity spectrum	Compounds	Reference
Streptococcus lactis C10	*Aspergillus parasiticus*	ND	Wiseman, D.W. and Marth, E.H., *Mycopathol.*, 1981, 73, 49.
Lactobacillus casei ATCC 393	*Aspergillus parasiticus*	ND	El-Gendy, S.M. and Marth, E.H., *J. Food Prot.*, 1981, **44**, 211.
L. casei var. *rhamnos*	Broad spectrum	ND	Coallier-Ascah, J. and Idziak, E.S., *Appl. Environ. Microbiol.*, 1985, **49**, 163.
L. casei var. *rhamnos*	Broad spectrum	<1 kDa	Vandenbergh, P.A. and King, S.W., 1988, EP 0,302,300,B1.
Lactobacillus reuteri	Broad spectrum	3-HPA (reuterin)	Talarico, T.L., Casas, I.A., Chung, T.C. and Dobrogosz, W.J., *Anti. Agents Chemo.*, 1988, **32**, 1854. Chung, T.C., Axelsson, L., Lindgren, S.E. and Dobrogosz, W.J., *Microbial Ecol. Health Dis.*, 1989, **2**, 137.
Lactobacillus plantarum	Unspecified spoilage mould	ND	Hill, J.E., 1989, US Patent Application 4,842,871.
S. lactis subsp. *diacetilactis* DRC1	*Aspergillus fumigatus* *Aspergillus parasiticus* *Rhizopus stolonifier*	Possibly proteinaceous	Batish, V.K., Grover, S. and Lal, R., *Cul. Dairy Prod. J.*, 1989, **24**, 21.
Lactobacillus acidophilus R	*Aspergillus fumigatus*	ND	Batish, V.K., Lal, R. and Grover, S., *Food Microbiol.*, 1990, **7**, 199.
Lactococcus lactis	*Aspergillus parasiticus*	ND	Luchese, R.H. and Harrigan, W.F., *J. Appl. Bacteriol.*, 1990, **69**, 512..
L. casei subsp. *rhamnosus* *L. plantarum* *Leuconostoc mesenteroides*	*Penicillium* spp. *Aspergillus* spp.	ND	Suzuki, I., Nomura, M. and Morichi, T., *Milchwissenschaft*, 1991, **46**, 635.
L. plantarum	*Saccharomyces cerevisiae*	ND	Makanjoula, D.B., Tymon, A. and Springham, D.G., *Enzyme Microb. Technol.*, 1992, **14**, 351.
L. casei subsp. *rhamnosus* LC-705	*Candida lusitaniae* *Aspergillus niger* *Fusarium* spp. *Penicillium* spp.	ND	Mäyrä-Mäkinen, A.K., Kristianinkatu, A. and Suomalainen, T.V., European Patent Application 0 576 780 A2.
L. lactis subsp. *lactis* CHD 28.3	*Aspergillus parasiticus* *Aspergillus flavus* *Fusarium* spp.	Possibly proteinaceous	Roy, U., Batish, V.K., Grover, S. and Neelakantan, S., *Inter. J. of Food Microbiol.*, 1996, **32**, 27.
L. casei	*Penicillium* spp.	Possibly proteinaceous	Gourama, H., *Lebensmittel Wissenschaft und Technologie*, 1997, **30**, 279.

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

LAB isolate*	Activity spectrum	Compounds	Reference
L. casei subsp. *pseudoplantarum*	*Aspergillus flavus*	Possibly proteinaceous <1 kDa	Gourama, H. and Bullerman, L.B., *Inter. J. of Food Microbiol.*, 1997, 34, 131. Gourama, H. and Bullerman, L.B., *J. of Food Prot.*, 1995, 58, 1249.
L. sanfrancisco CB1	*Fusarium* spp. *Penicillium* spp. *Aspergillus* spp. *Monilia* spp.	Caproic acid Propionic acid Butyric acid Valeric acid	Corsetti, A., Gobebetti, M., Rossi, J. and Damiani, P., *Appl. Microbiol. Biotechnol.*, 1998, **50**, 253–256.
L. plantarum VTT E78076	*Fusarium avenaceum*	Benzoic acid, methylhydantoin, mevalonolactone, cyclo (Gly-L-Leu)	Niku-Paavola, M.-L., Laitila, L., Mattila-Sandholm, T. and Haikara, A., *J. Appl. Microbiol.*, 1999, **86**, 29.
L. pentosus	*Candida albicans*	Pentocin TV35b	Okkers, D.J., Dicks, L.M.T., Silvester, M., Joubert, J.J and Odendaal, H.J., *J. Appl. Microbiol.*, 1999, **87**, 726.
L. casei *L. delbreuckii* subsp. *bulgaricus*	*Penicillium expansum*	ND	Florianowicz, T., *Eur. Food. Res. Technol.*, 2001, **212**, 282.
L. plantarum	Broad spectrum	Phenyllactic acid 4-hydroxyphenyllactic acid	Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. and Gobetti, M., *Appl. Environ. Microbiol.*, 2000, **66**, 4084.
L. rhamnosus	*Fusarium* spp. *Penicillium* spp. *Aspergillus* spp. *Alternaria* spp.	Sodium acetate[1]	Stiles, J., Penkar, S., Plockova, N., Chumchalova, J. and Bullerman, L.B., *J. Food Prot.*, 2002, **65**, 1188.
L. plantarum MiLab 393	Broad spectrum	3-Phenyllactic acid cyclo(Phe-Pro) cyclo(Phe-OH-Pro)	Ström, K., Sjögren, J., Broberg, A. and Schnurer, J., *Appl. Environ. Microbiol.*, 2002, **68**, 4322.
L. coryniformis Si3	Broad spectrum	Peptide Phenyllactic acid cyclo(Phe-Pro) cyclo(Phe-OH-Pro)	Magnusson, J. and Schnürer, J., *Appl. Environ. Microbiol.*, 2001, **67**, 1. Magnusson, J., Ström, K., Roos, S., Sjögren, J. and Schnürer, J., *FEMS Microbiology Letters*, 2003, **219**, 129.
L. plantarum MiLab 14	Broad spectrum	Hydroxy fatty acids Phenyllactic acid cyclo (Phe-Pro) cyclo (Phe-OH-Pro)	Sjogren, J., Magnusson, J., Broberg, A., Schnurer, J. and Kenne, L., *Appl. Environ. Microbiol.*, 2003, **69**, 7554. Magnusson, J. and Schnürer, J., *Appl. Environ. Microbiol.*, 2001, **67**, 1. Magnusson, J., Ström, K., Roos, S., Sjögren, J. and Schnürer, J., *FEMS Microbiology Letters*, 2003, **219**, 129.

[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.

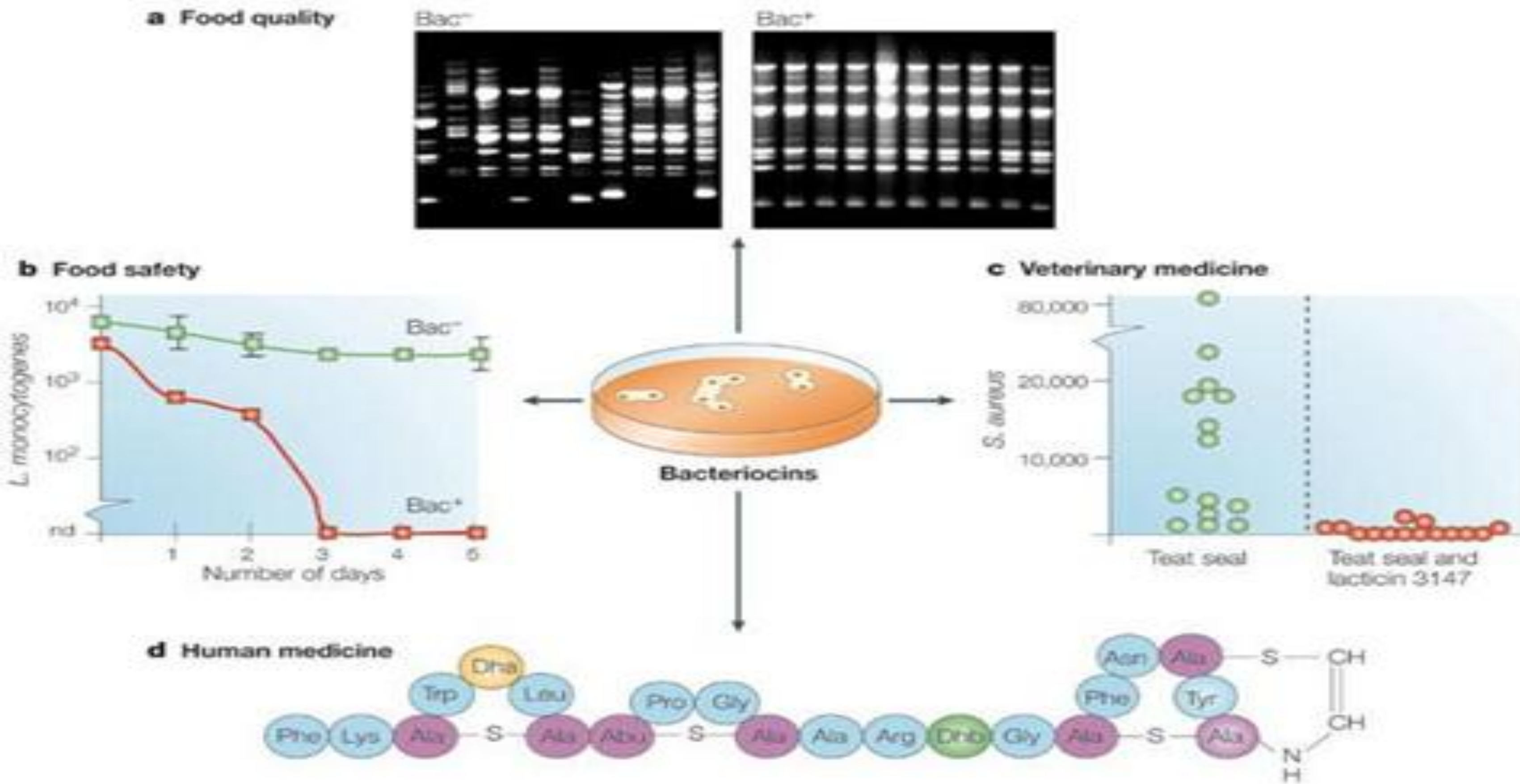
Bacteriocin producing protective cultures

Table 1 | **Suggested classification scheme for bacteriocins**

Classification*	Remarks/suggestions	Examples
Class I		
Lanthionine-containing bacteriocins/lantibiotics	Includes both single- and two-peptide lantibiotics; up to 11 subclasses have been proposed[19]	Single-peptide: nisin, mersacidin, lacticin 481; two-peptide: lacticin 3147, cytolysin
Class II		
Non-lanthionine-containing bacteriocins	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)	Class IIa: pediocin PA1, leucocin A; class IIb: lactacin F; class IIc: enterocin AS48, reuterin 6; class IId: lactococcin A, divergicin A
Bacteriolysins		
Non-bacteriocin lytic proteins‡	Large, heat-labile proteins, often murein hydrolases	Lysostaphin, enterolysin A

* Class IV bacteriocins (bacteriocins with non-proteinaceious moieties) are not included as no members have been demonstrated.
‡ Suggested that these are no longer considered bacteriocins (see main text).

Bacteriocin based «protection»



CLERICI SACCO

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

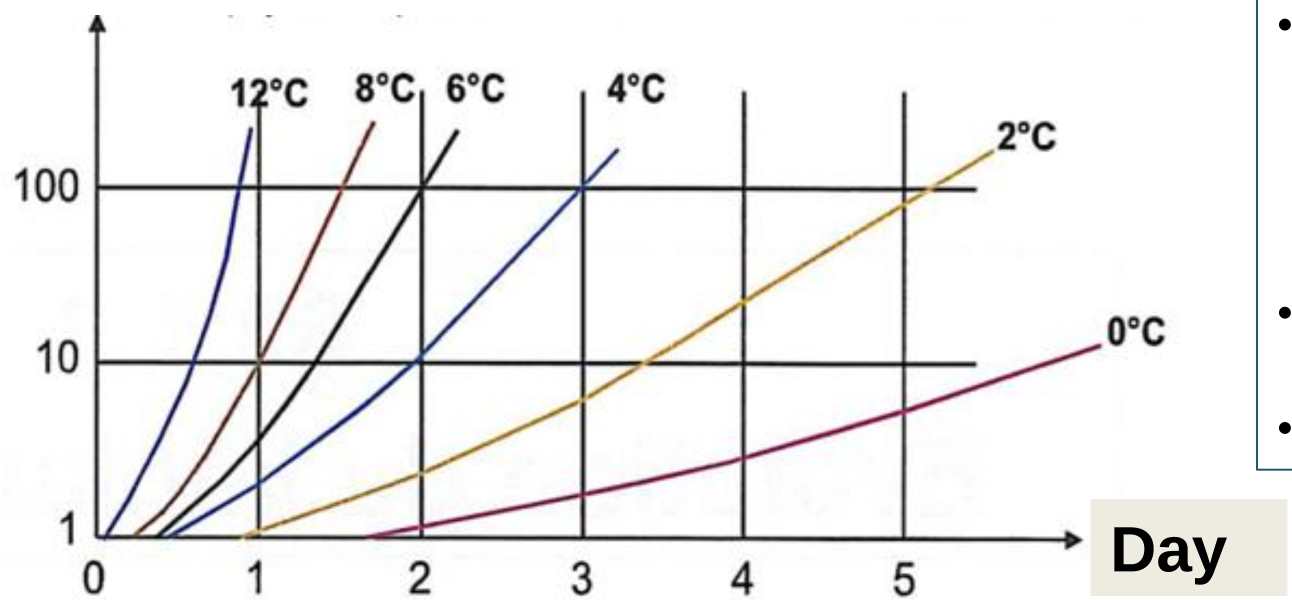
Raw milk biota development

Psychrotrophic bacteria :

About 10% of the psychrotrophic bacteria in the milk are **pseudomonas . (Problem: count > 10.000 CFU)**

Diagram of growth

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

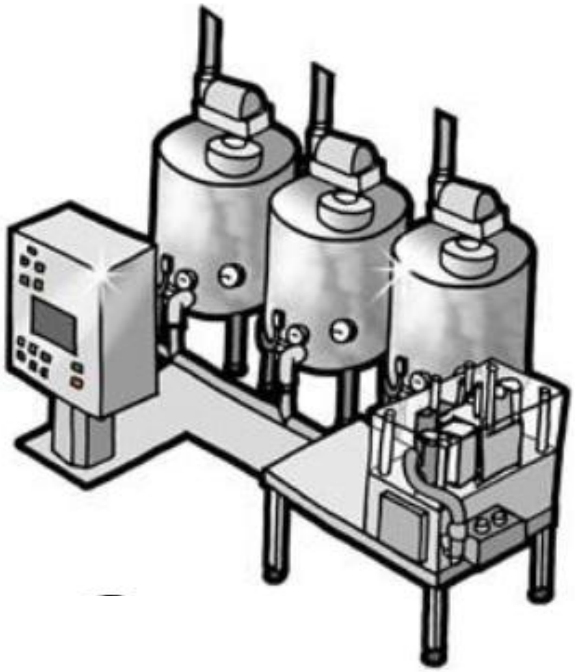


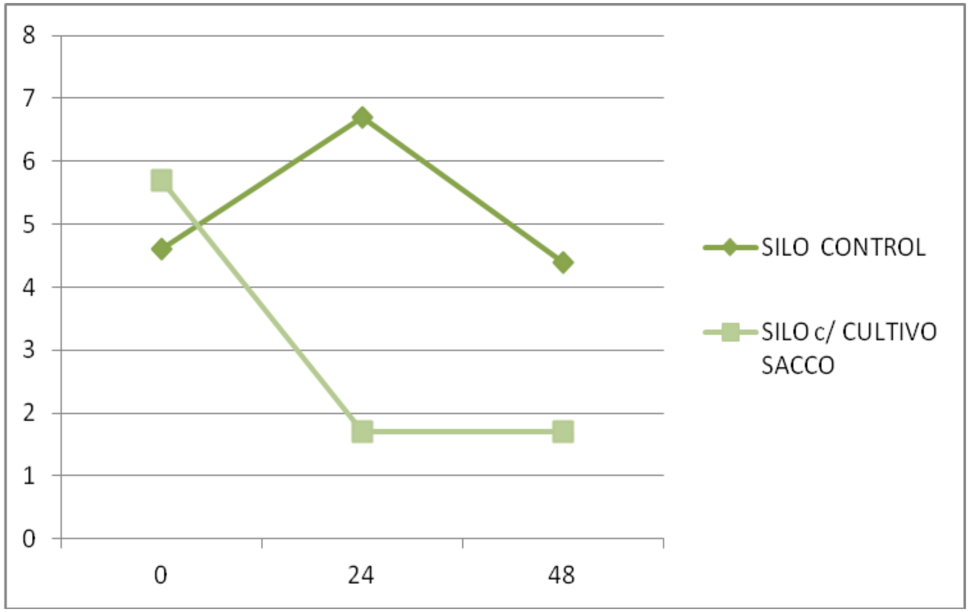
- **Able to produce** exocellular and thermoresistent enzymes!!
- **Lypases**
- **Protease**

Source: ENIL BIO - 2007

Raw milk «protection»
ex 1. LR B - 5x10E4/ml

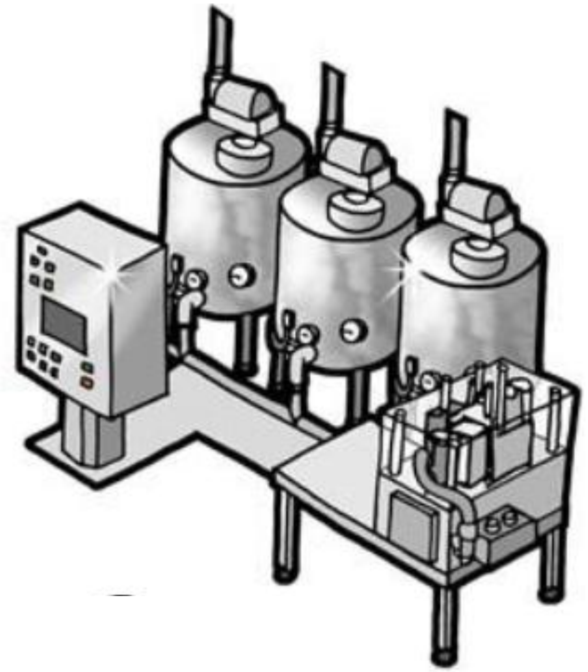
Time in hours	MESOPHILIC AEROBIC BACTERIA x 10^6	
	SILO CONTROL	SILO c/ LRB
0	4,6	5,7
24	6,7	1,7
48	4,4	1,7





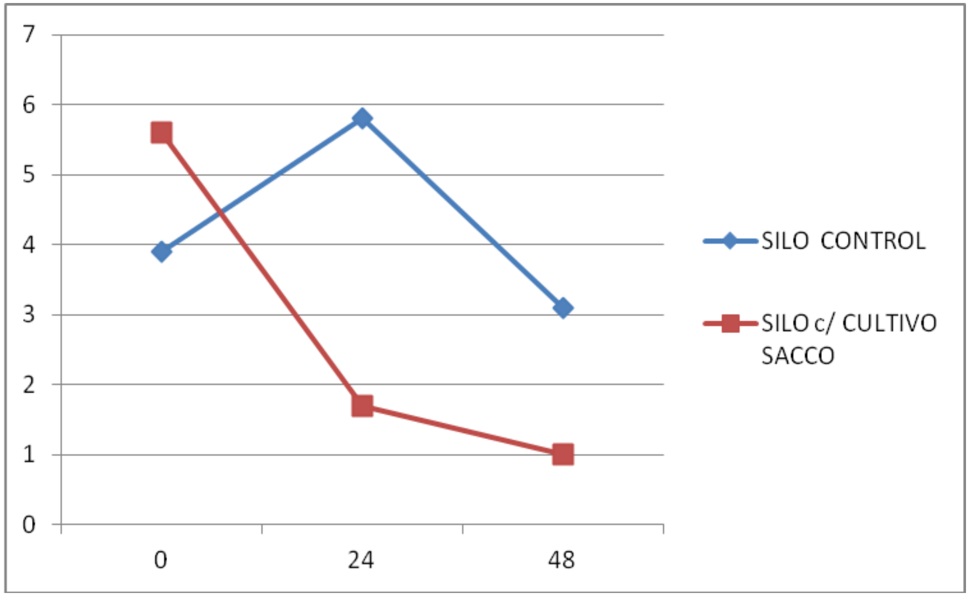
Time of action LR B
min 7-8 h

Raw milk «protection»
ex 1. LR B - 5x10E4/ml

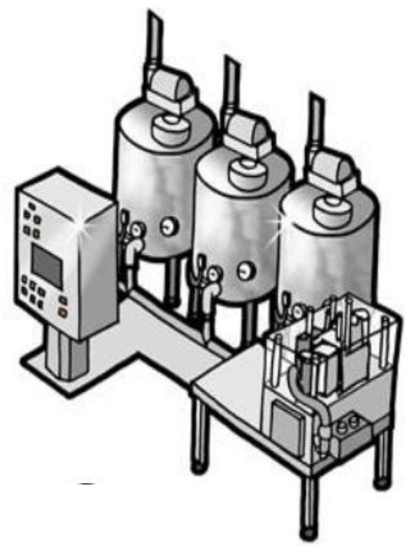


Time of action LR B min 7-8 h

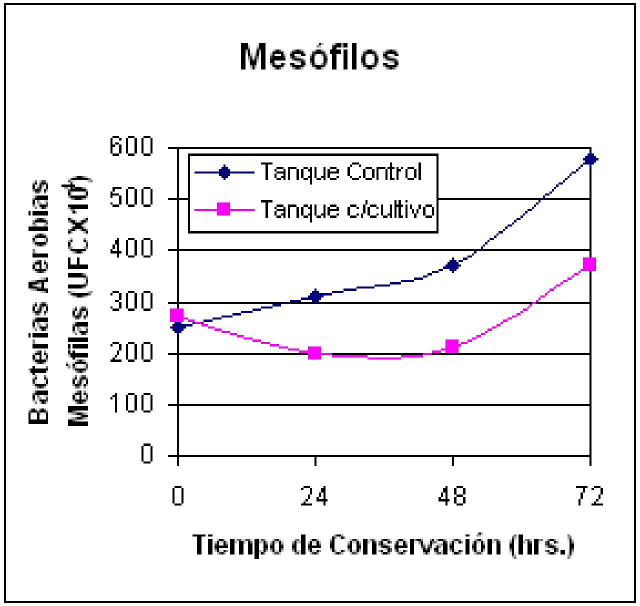
Time in hours	PSYCHROTROPHIC BACTERIA x 10^6	
	SILO CONTROL	SILO / LRB
0	3,9	5,6
24	5,8	1,7
48	3,1	1

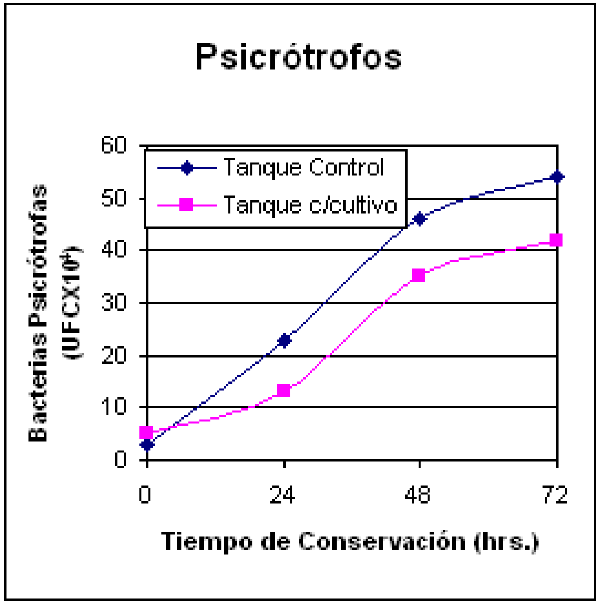


Raw milk «protection»
ex 2. LR B - 5x10E4/ml



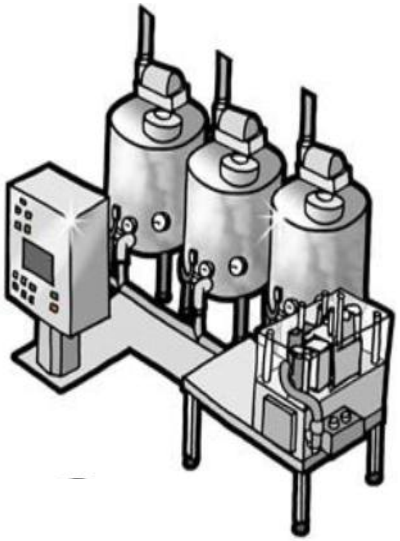
Fecha	Tiempo de conservación (hrs.)	Mesófilos x 10^4		Psicrótrofos x 10^4	
		Tk Control	Tk c/cultivo	Tk Control	Tk c/cultivo
07/04/2014	0	250	270	3	5
08/04/2014	24	310	200	23	13
09/04/2014	48	370	210	46	35
10/04/2014	72	580	370	54	42





Time of action LR B
72 h

Raw Cream «protection»



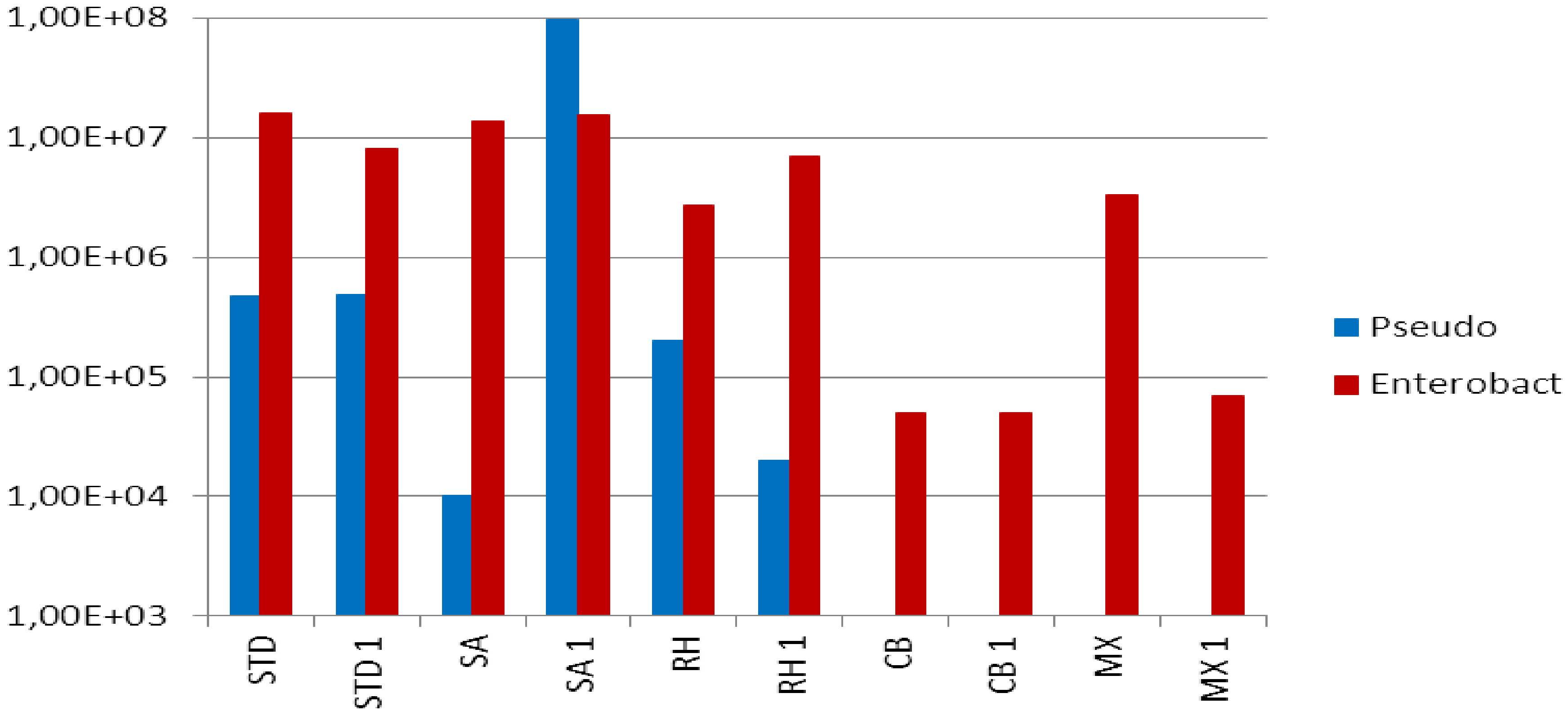
		AEROBIC MESOPHILIC BACTERIA	PSYCHROTROPHIC BACTERIA
Test 1	Raw cream without LR B	44.000	3.000
	Raw cream with LR B	6.100	100
Test 2	Raw cream without LR B	233.000	124.000
	Raw cream with LR B	222.000	79.000
Test 3	Raw cream without LR B	920.000	8.800
	Raw cream with LR B	180.000	8.000

Fresh cheese «protection»

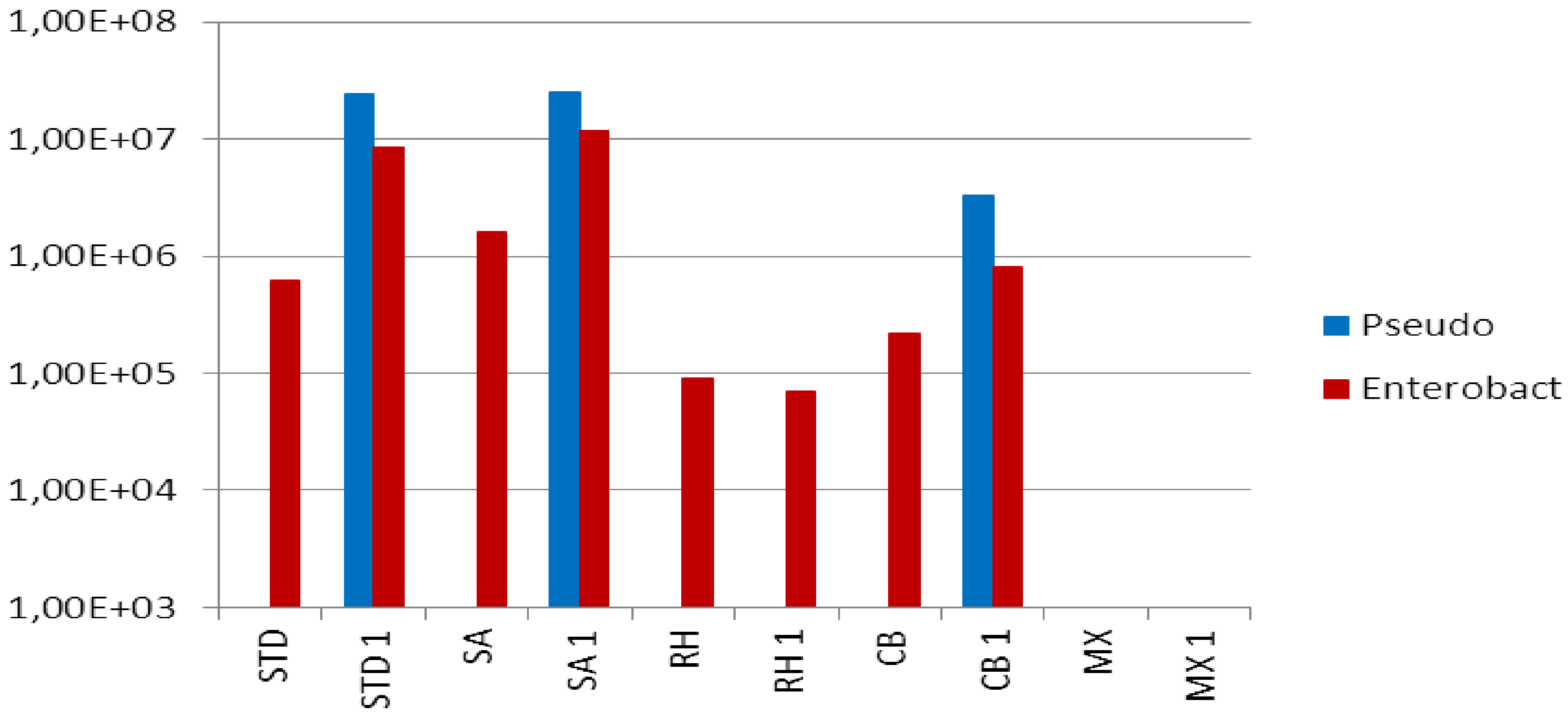
- Fresh and soft not matured and weakly fermented cheese types with high pH (from above 6 and down to 5) have issues with out growth of unwanted microorganism

- 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



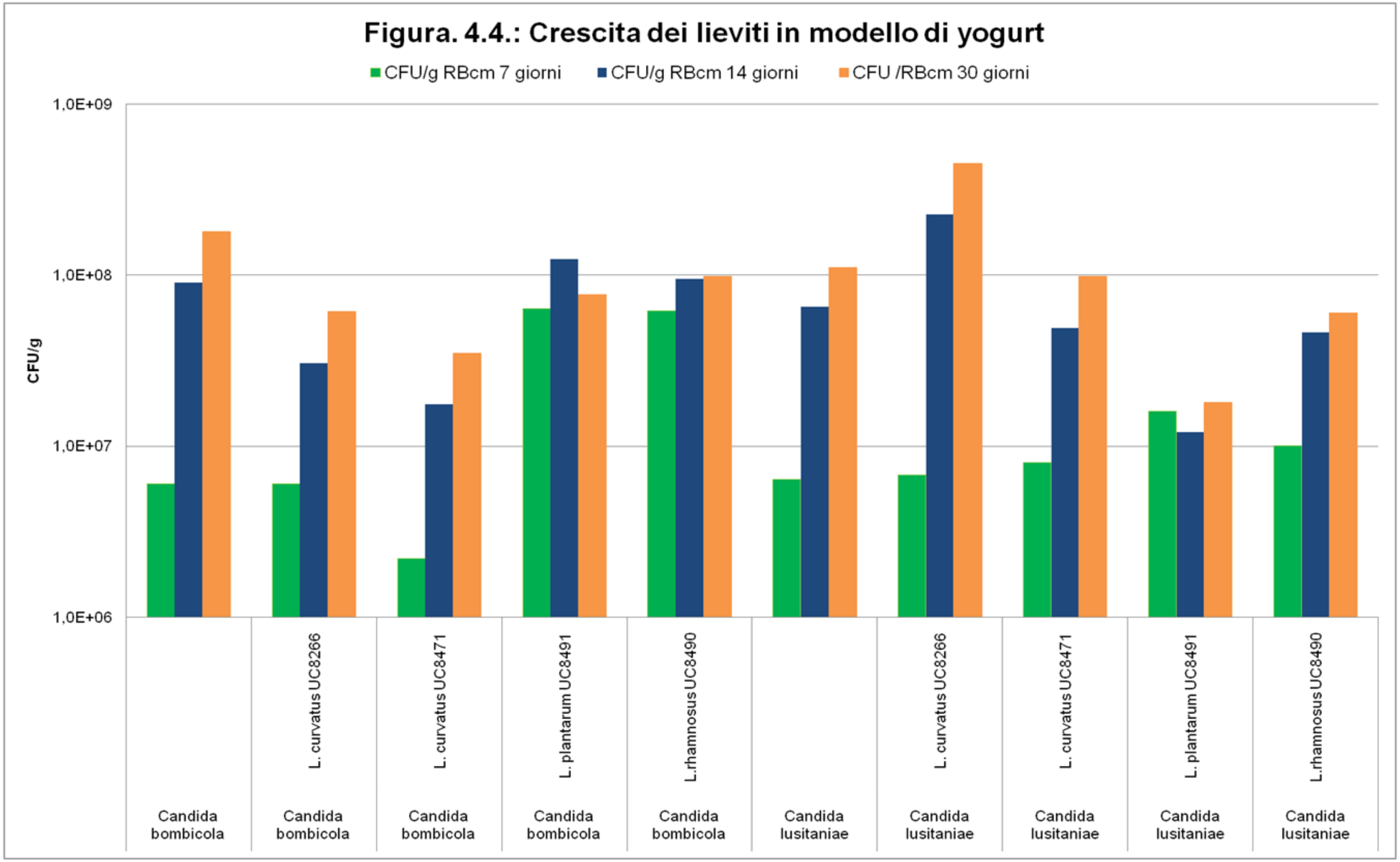
Fresh cheese «protection»
discussion of results

- *Lactobacillus* (LRH) and/or Carnobact. 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

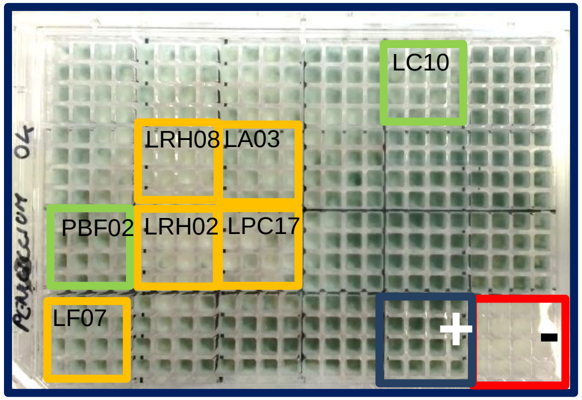
Microrganisms used


Contaminant	Internal code
Penicillium roqueforti	PRN + PRA
Kluyveromyces marxianus	KLM02
Saccharomyces cerevisiae	SCH03
Debaryomyces hansenii	DH01

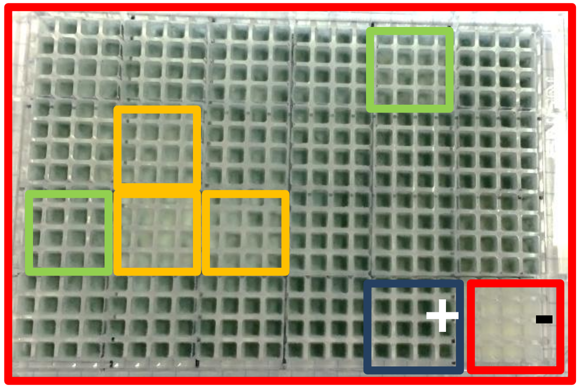
LAB «protection» against moulds

PR on yoghurt inhibited by different strains

\+ *Penicillium*



\+ *Penicillium* + yeasts



Penicillium inoculated 10^5 spore/ml
Yeasts (blend of DH, KLM, SC)10^2 CFU/ml

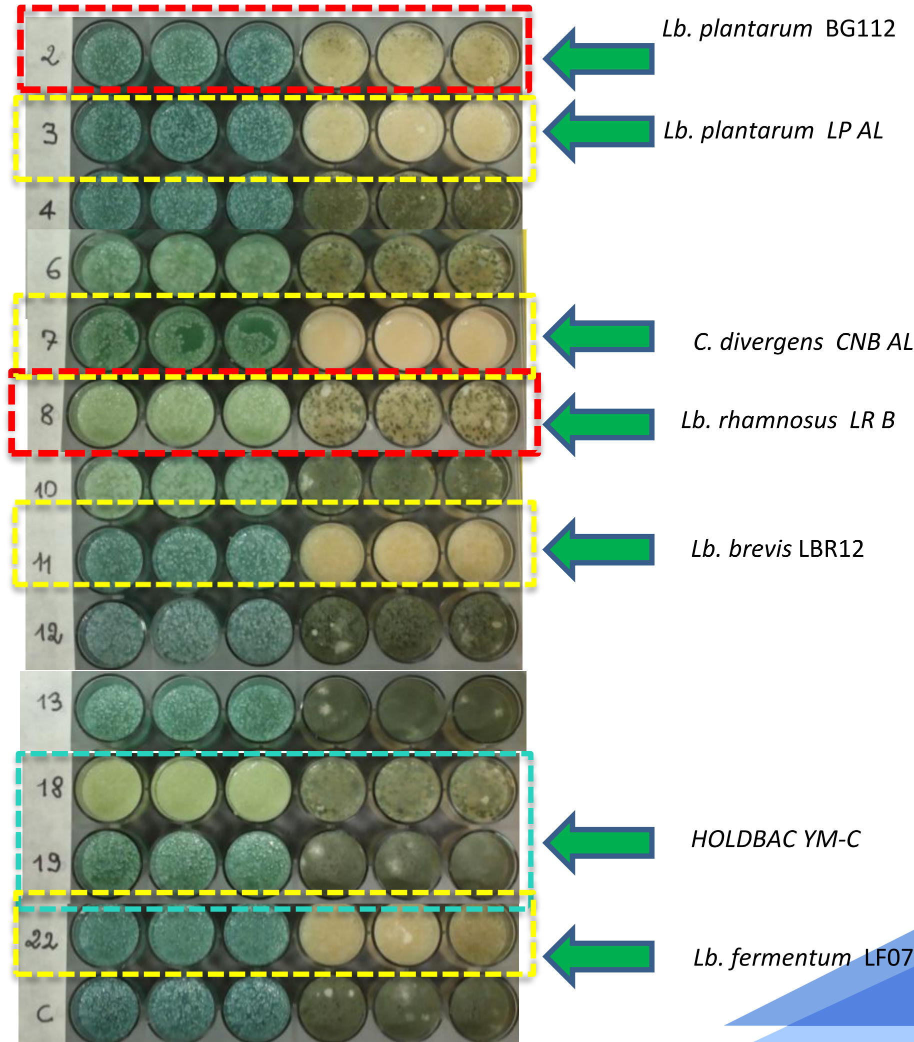
After 10 days of incubation at 10 °C

The best *Penicillium* sporulation-inhibitor are: LRH08, LA03, **LRH02**, **LPC17,** **LF07.**
Weak inhibition for PBF02, LC10

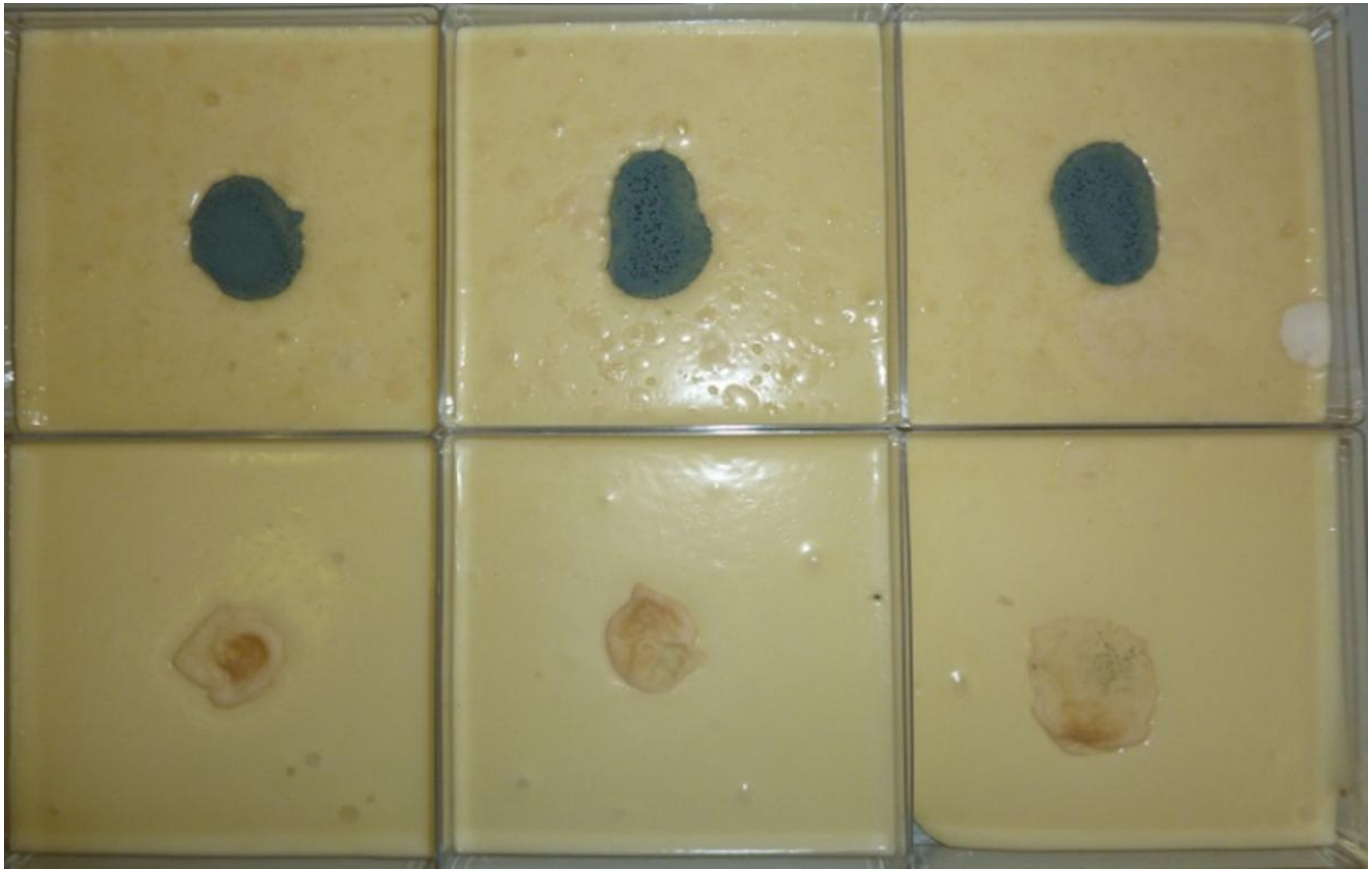
Anti Y&M screening

10^4 CFU/ml yeast + 10^4 CFU/ml mould
Incubation : 6 days

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

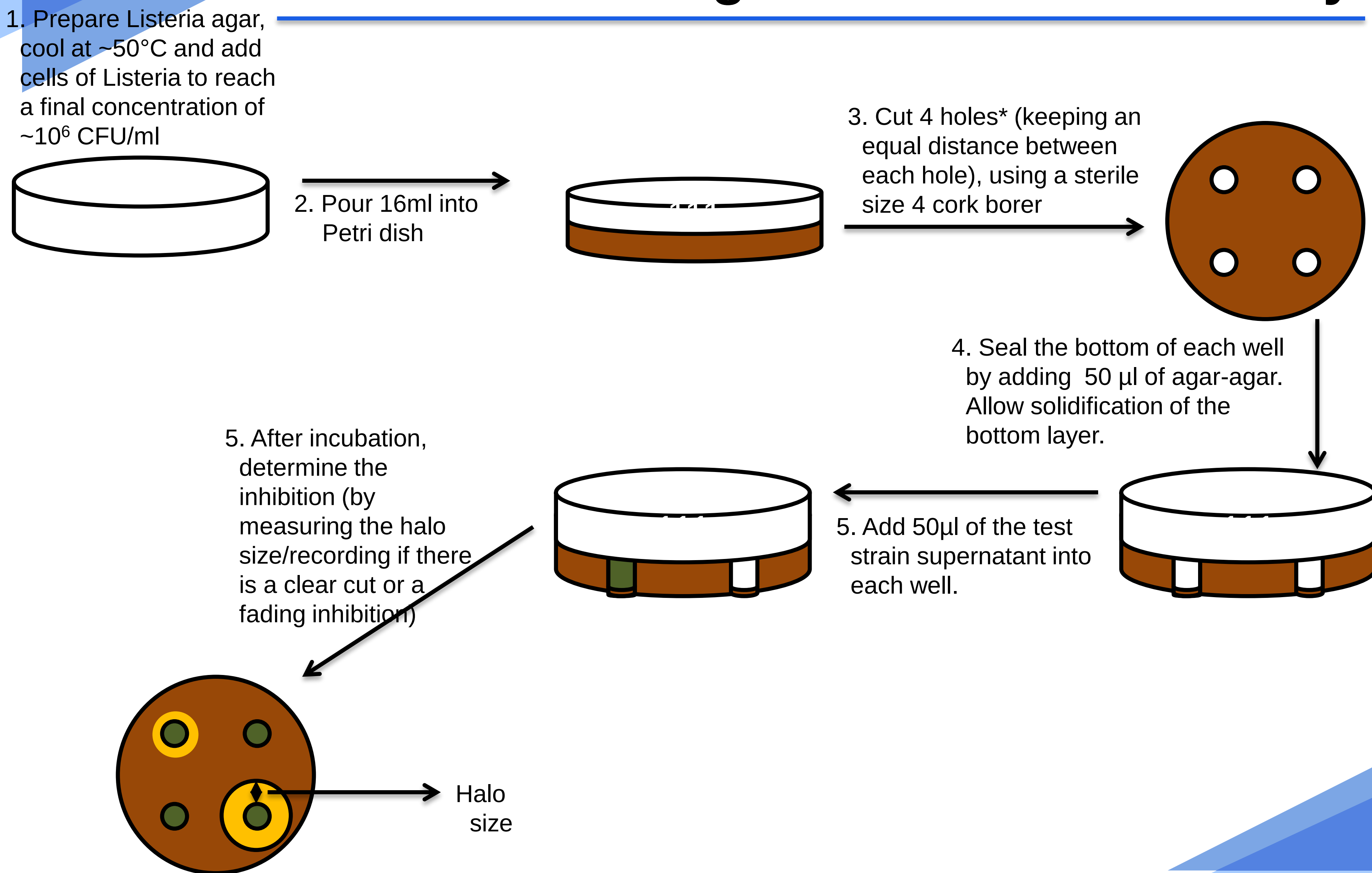


Example of anti-mould activity on model cheese

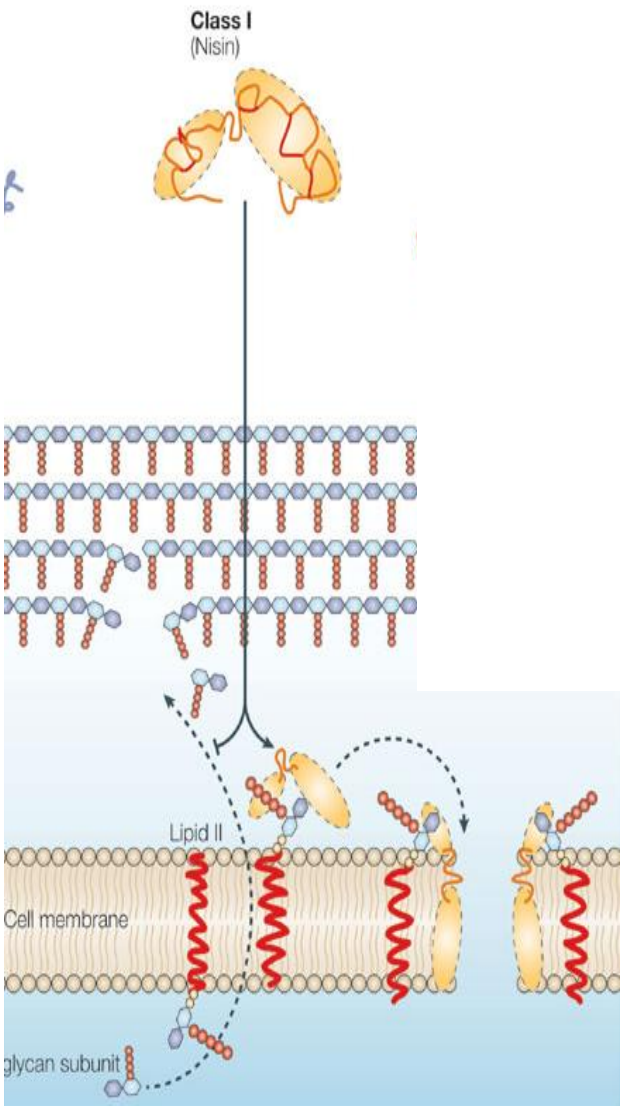


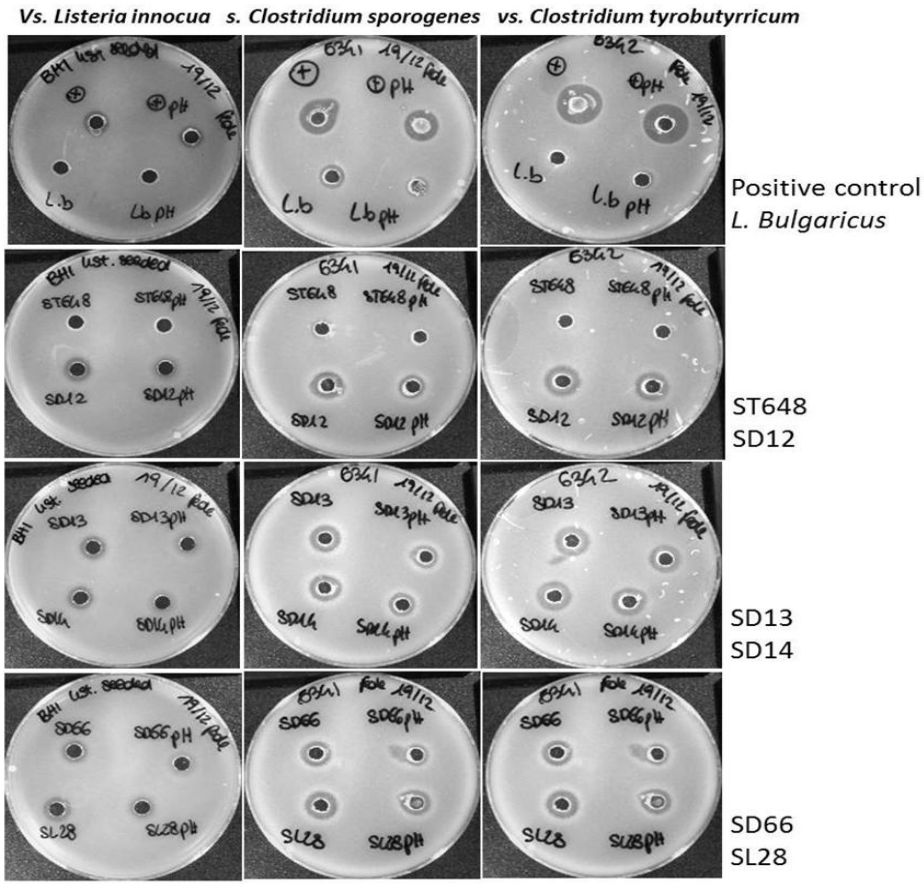
Example of cheese inoculated with LRB

Agar well diffusion assay



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 tretaments (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!

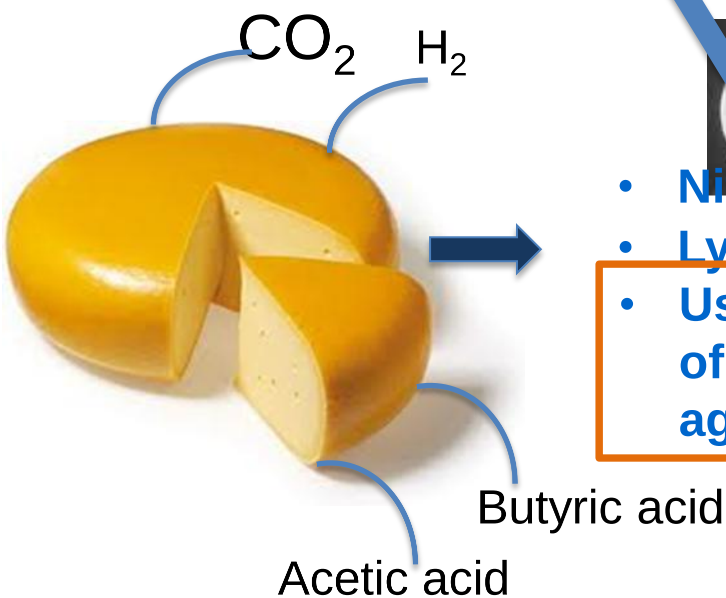
Anti-Listeria activity in Gorgonzola

Example 2

Cheese surface treated with *Carnobacterium* salt solution

➔ Also effective against surface foreign moulds growth

Cheese «Protection» against Clostridia



cheese blowing

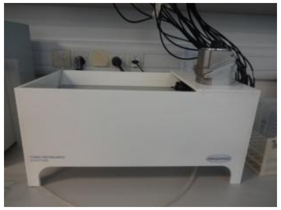
- Nitrates addition
- Lysozyme addition
- Use of a strain producer of a bacteriocin active against Clostridia

Bac$^+$ producers in a mini-cheese

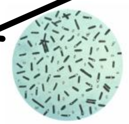
Gouda cheese production in laboratory scale

Industrial scale

Lab scale



1. Inoculum: 30 min



- $CaCl_2$
- Starters
- *Cl. tyrobutyricum* spores
- +/- bacteriocin producer strain/s

2. Renneting and curd cutting: 45 min

Bac+ producers in a mini-cheese

Industrial scale

Lab scale

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

Stirring in vat

Low speed centrifugation

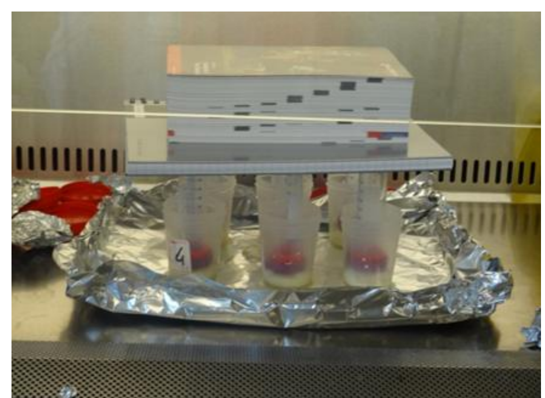
4. Stirring, heating and hot water addition: 40 min

5. Whey off: 60 min

Pressing systems

Low speed centrifugation

6. Pressing: 30 min

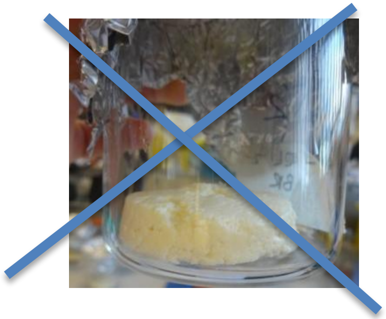


Bac⁺ producers in a mini-cheese

Industrial scale

Lab scale

7. Brining: 0 (to simulate cheese core)



8. Vacuum pack

10Kg vs. 15g

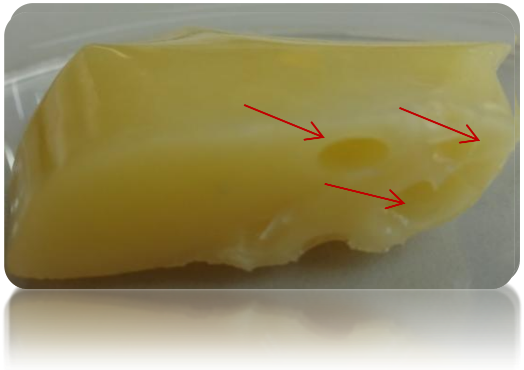
9. Ripening

~11-18°C

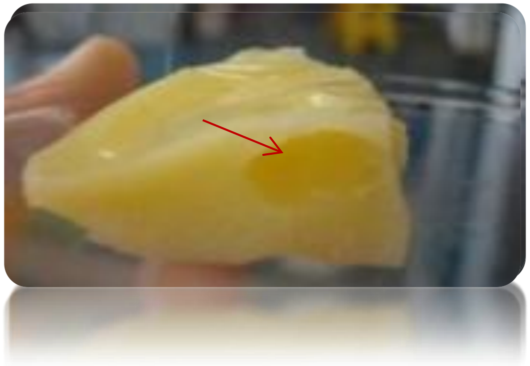
~20°C (to be sure!)

Bac^{+} producers in a mini-cheese

<u>Sampling and cheese analysis:</u>



1 month ripening, no bacteriocin producer added

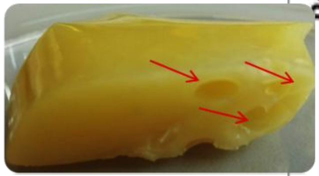


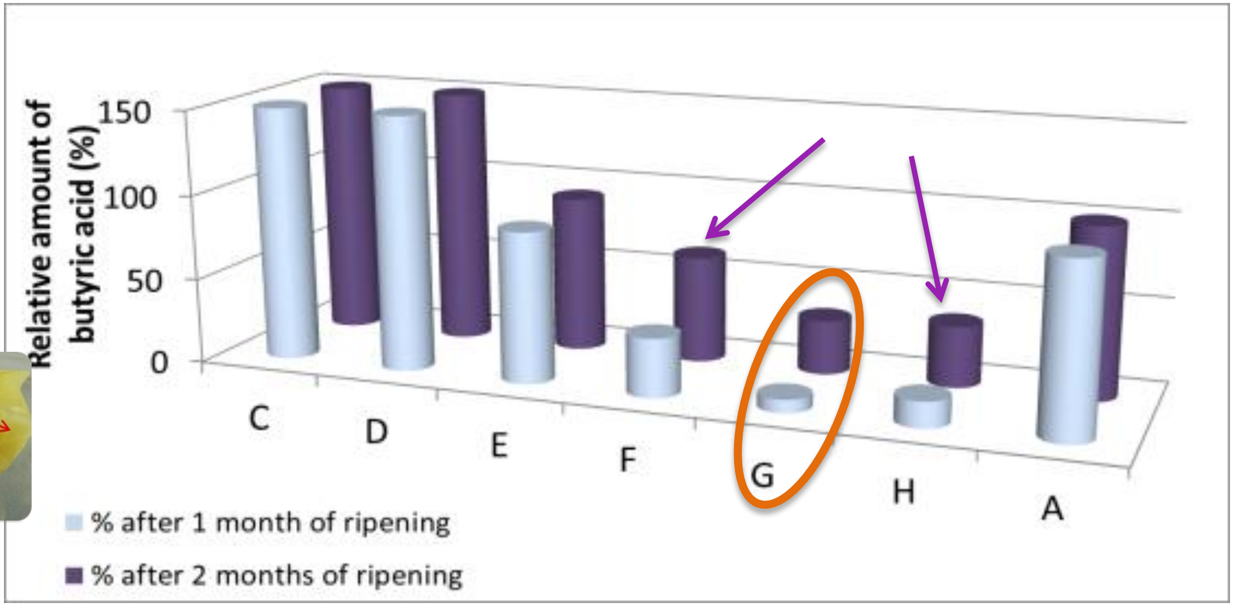
- samples collected after 4 and 8 weeks of ripening;
- free fatty acid extraction (FFA) and Gas Chromatography analysis for the detection and the quantification of the butyric acid;
- well diffusion assay of small portion of cheese;

Bac$^+$ producers in a mini-cheese

Mini-cheese added with <u>single</u> 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%)

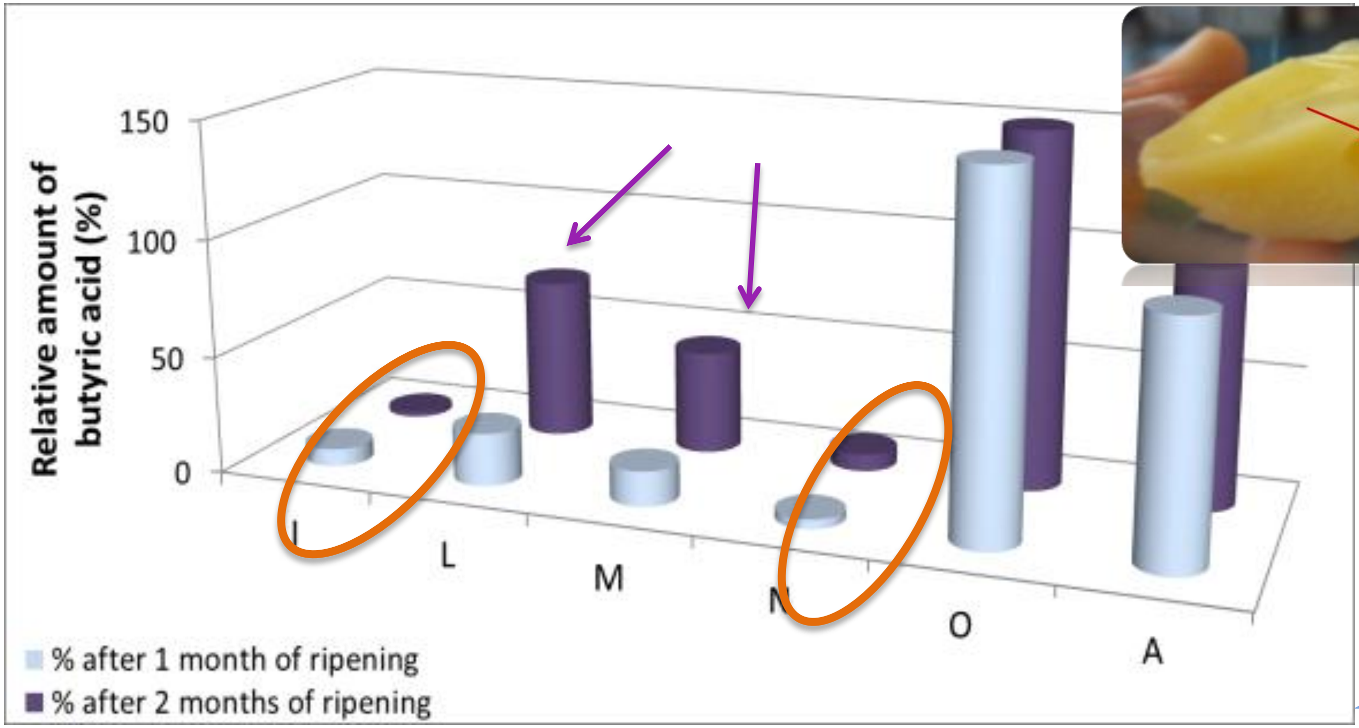


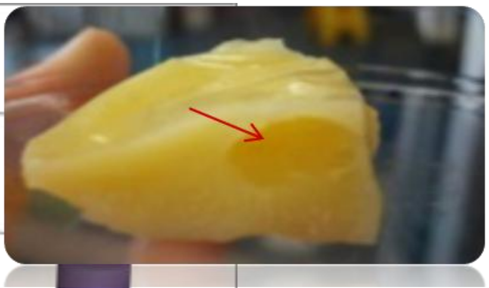


Bac+ producers in a mini-cheese

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%)





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

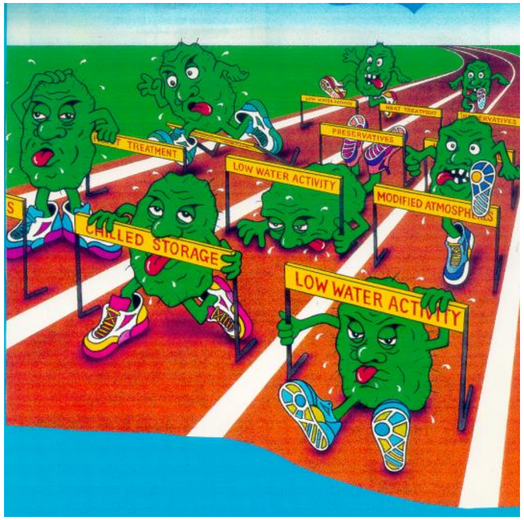
NSLAB used for Cheese «Protection» against late blowing

NSLAB used for Cheese «Protection» against late blowing

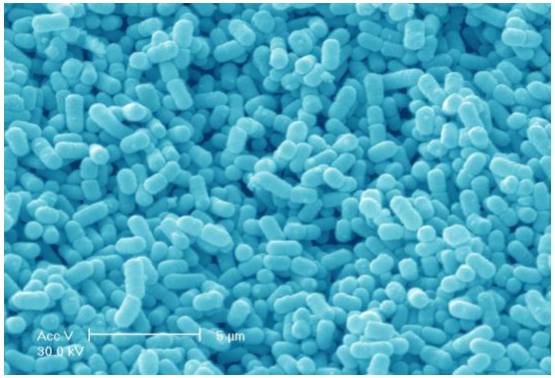
NSLAB used for Cheese «Protection» against late blowing

Other meat products

- 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 2ºC
  - 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 2ºC
  - Useful in all meat and fish applications
  - Not a strong competitor
  - Only for enhanced safety
  - No acidification
  - No sensory impact

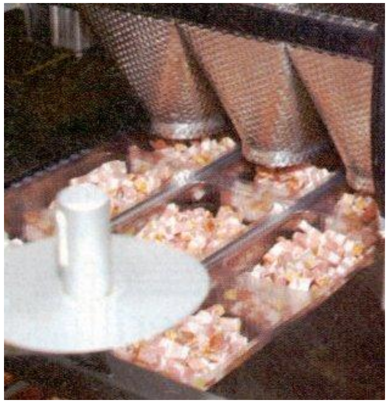
- On license from French University
  - Documentation available
    - Efficient against 57 *L. monocytogenes* strains

Protective 1: BOM-13, BOX-74, FP-18

- Meat <u>without nitrite</u> 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

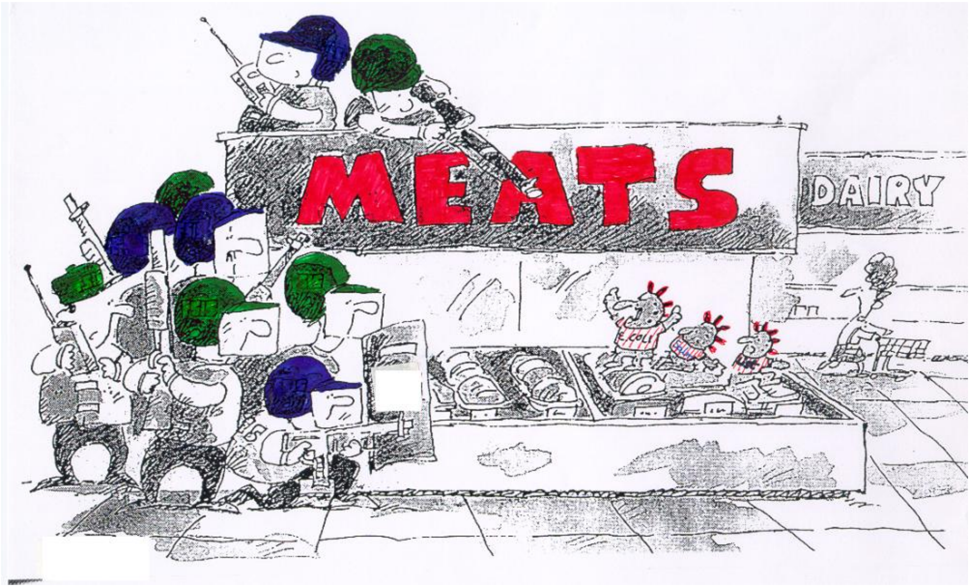


- Fresh meat products <u>with nitrite</u> applied packed in vacuum or MAP
  - Lyocarni BXH-69 with *Lb. sakei*
  - Lyocarni BMX-37 with *Lb. sakei* and *Carnobacterium*
  - Both with added Staphylococci

- 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 of initial bacterial load. Recommendable >$5x10^5$ 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

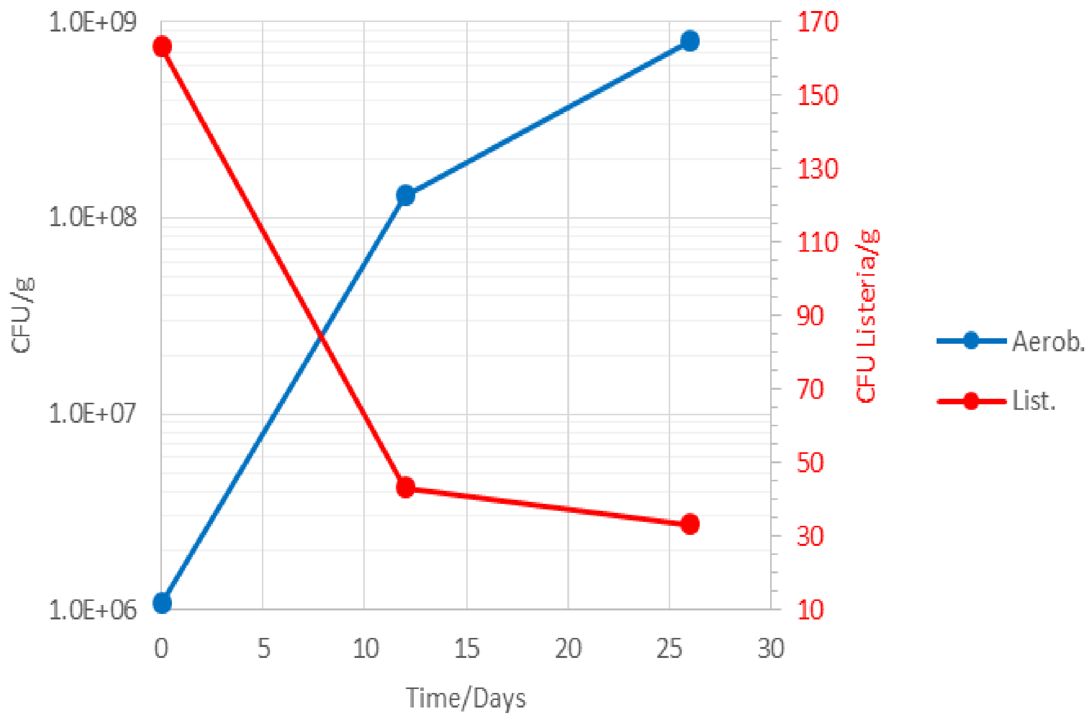


Application of protective culture

Type of meat	How to apply
Fresh minced meat	Sprinkled into the mince
- Whole pieces	Spray (or dip) on surface
- Injected brine	Dissolve in brine
Frozen meat	Apply before freezing and it will work when the product is thawed
Processed meat	Spray (or dip) it on surface after heat treatment and cooling

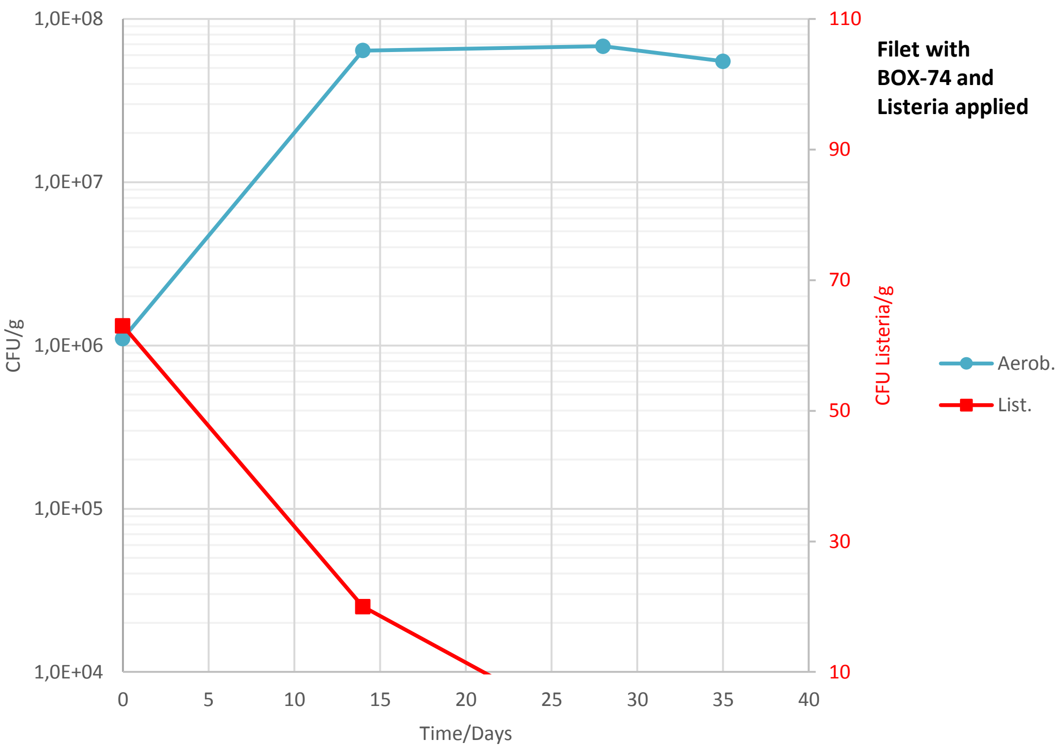
Challenge test 1: cooked ham

- Smoked, cooked, sliced ham
  - Lyocarni BOX-74
    - Sprayed on during slicing
  - MAP
  - Stored at 8ºC
  - Control initially
    - $1.7x10^3$ CFU aerobic bacteria/g
    - No *L. monocytogenes* detected in 25 g



Challenge test 2: cold-smoked filet

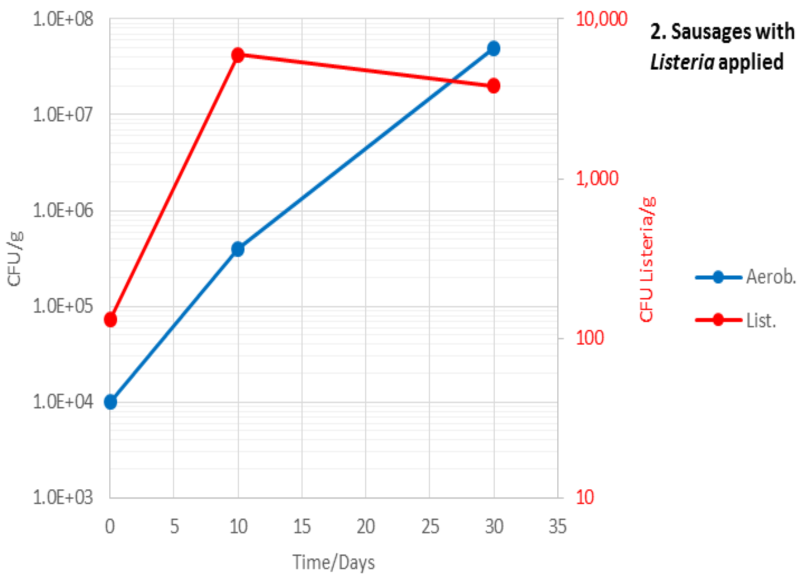
- Cured, cold-smoked, sliced filet
  - Lyocarni BOX-74
    - Sprayed on during slicing
  - MAP
  - Stored at 5ºC
  - Control initially
    - $<10^4$ CFU aerobic bacteria/g
    - No *L. monocytogenes* detected in 25 g

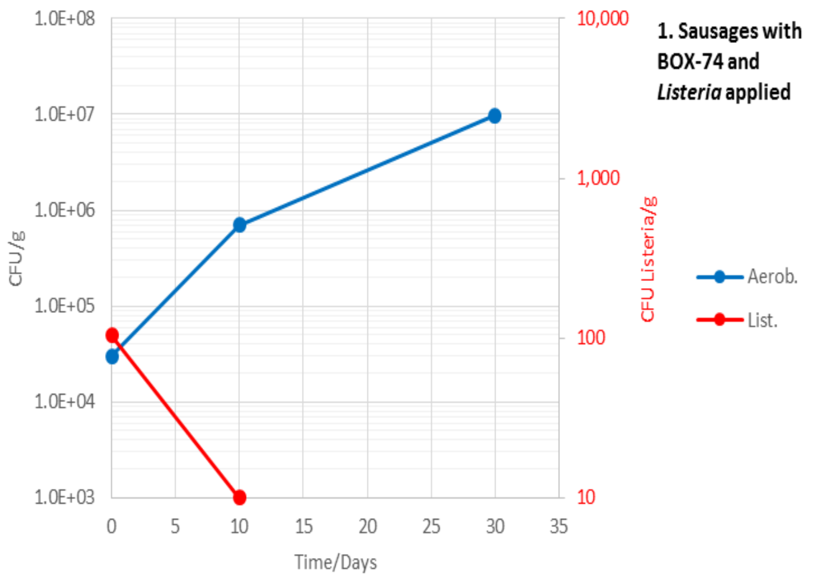


Challenge test 3: emulsion sausages 1

- Cooked emulsion sausages
  - Lyocarni BOX-74
    - Cooling and removal of skin
    - Sprayed on before packaging
  - MAP
  - Stored at 8ºC
  - Control initially
    - 3.3×10^3 CFU aerobic bacteria/g
    - No *L. monocytogenes* detected in 25 g

Challenge test 3: emulsion sausages 2

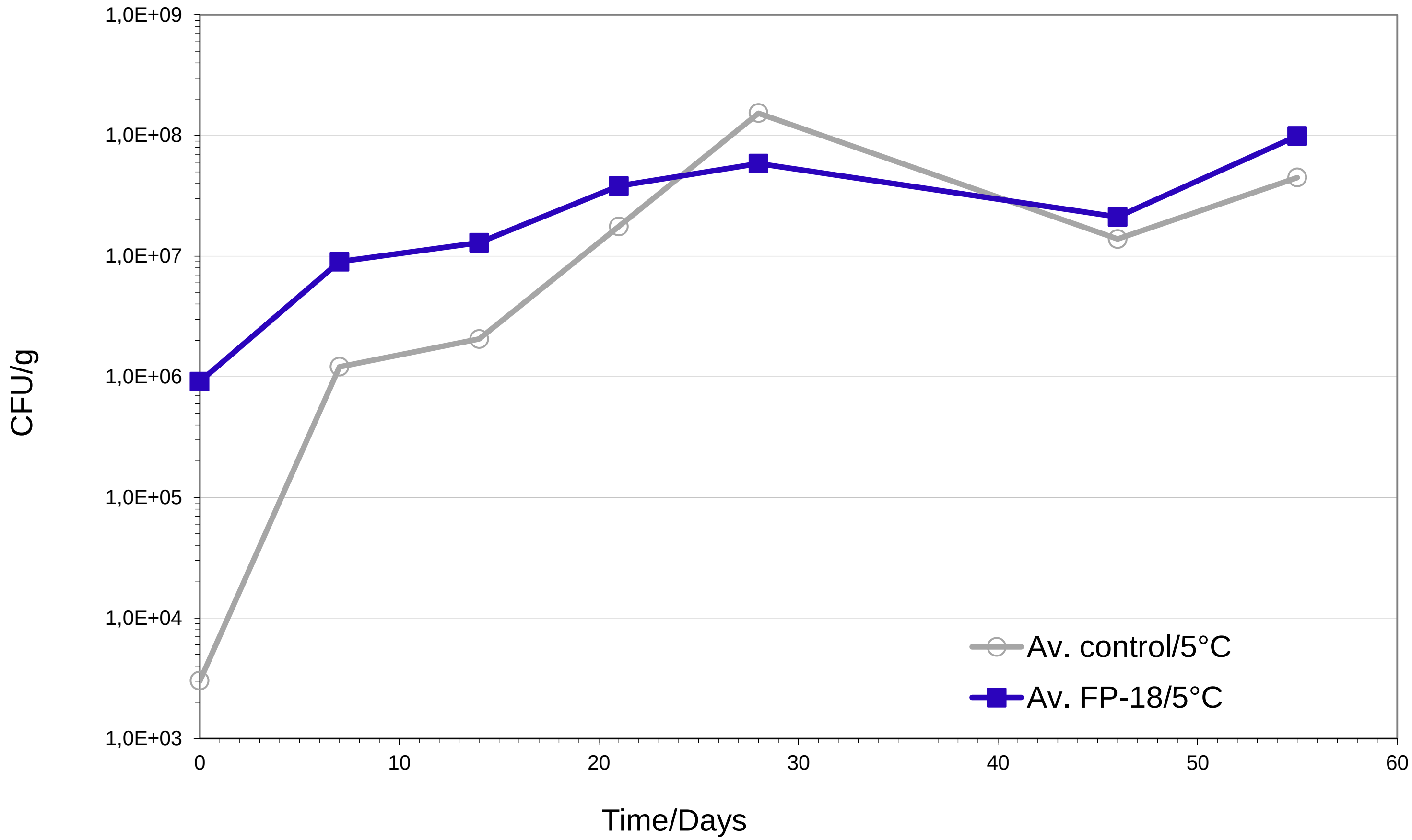




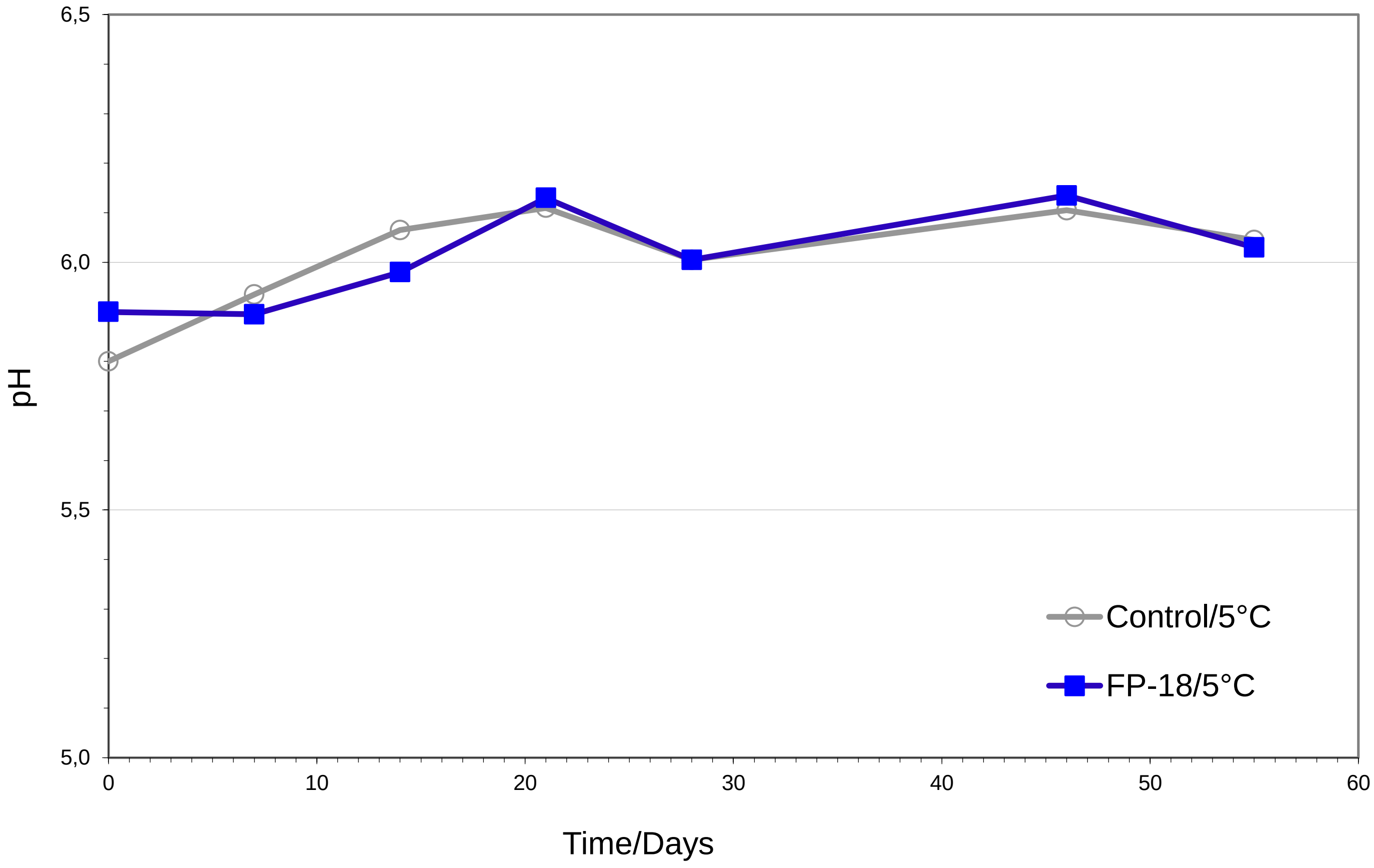
Fish matrix

- 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

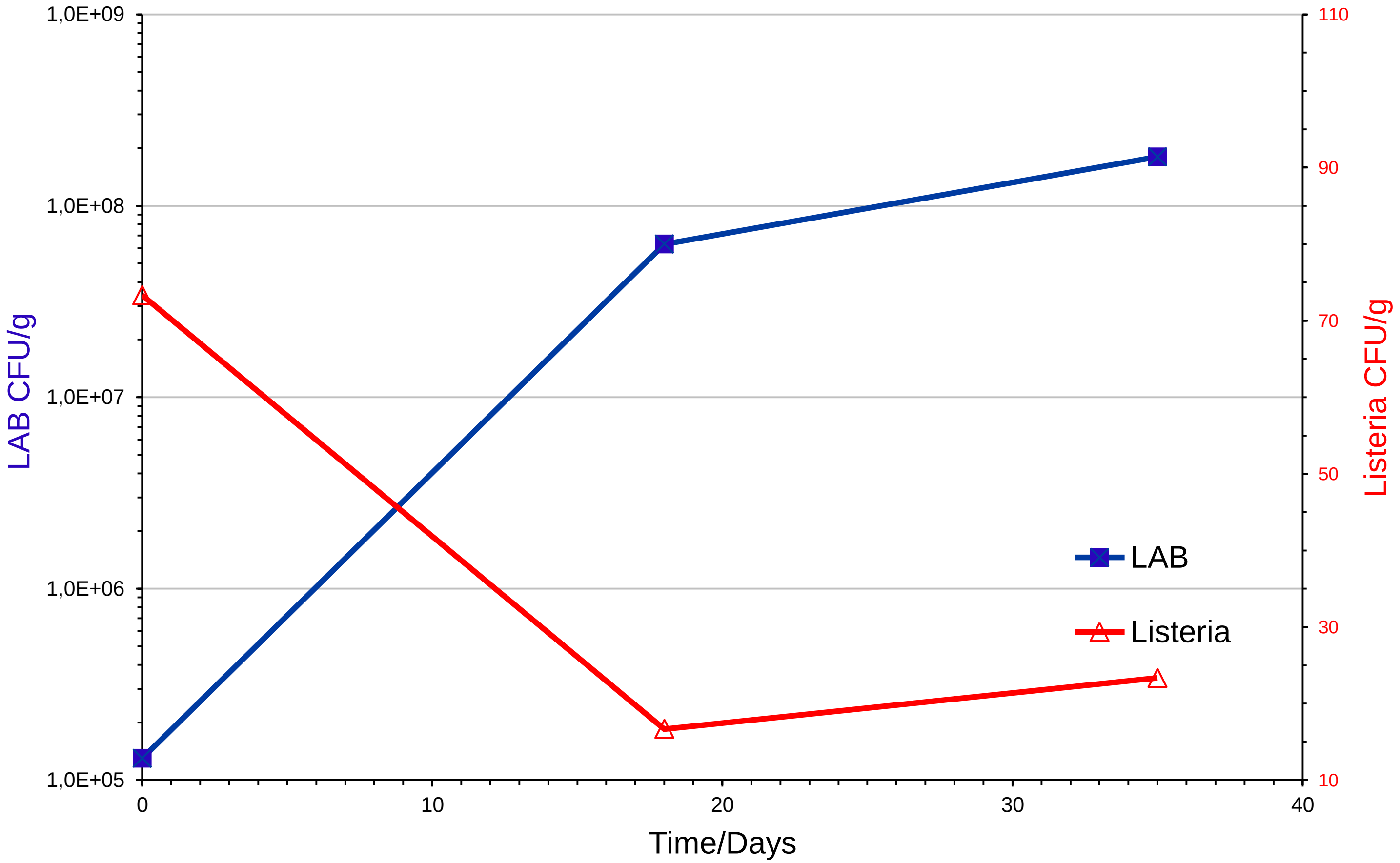
LAB growth in smoked salmon



pH in smoked salmon



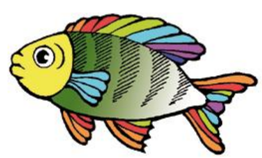
Challenge test: smoked salmon at 5ºC



Application of Lyoflora FP-18

- Application examples (aiming at $>10^5$ CFU/g)
  - **Injection**: with 3.5% injected weight gain; 3.5 kg brine with 5.2×10^{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?

Clerici-Sacco Group

Sacco S.r.l. www.saccosrl.it

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