HELSINGIN YLIOPISTO

TRENDS OF ANTIBIOTIC RESISTANCE IN BACTERIAL POPULATIONS DURING COLD STORAGE OF RAW MILK FROM CONVENTIONAL VERSUS ORGANIC FARMING SYSTEMS

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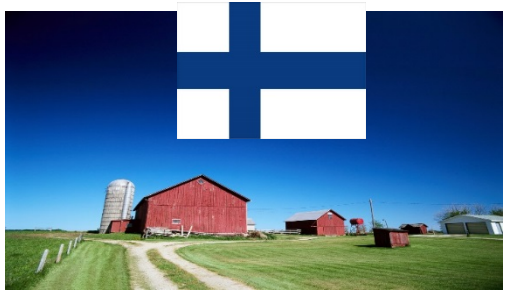
MIKKELI, 28th of October 2014

WHAT IS RAW MILK?

Milk that has not been subjected to any heat treatment

ORGANIC COMPARED TO CONVENTIONAL FARMING SYSTEMS (EU ORGANIC REGULATIONS)

Animals should have more living space, regular outdoor access, be fed mostly on forage (until 2008: up to 5% from conventional sources was allowed); prophylactic use of antibiotics is prohibited; antibiotics may be used for disease treatments (then the withdrawal time is prolonged)



RAW MILK PRODUCTION IN FINLAND

Conventional farming

*10450 dairy farms in 2011
(TIKE & Evira)

Organic farming

*147 dairy farms in 2011
*1,4% total raw milk production (Evira)

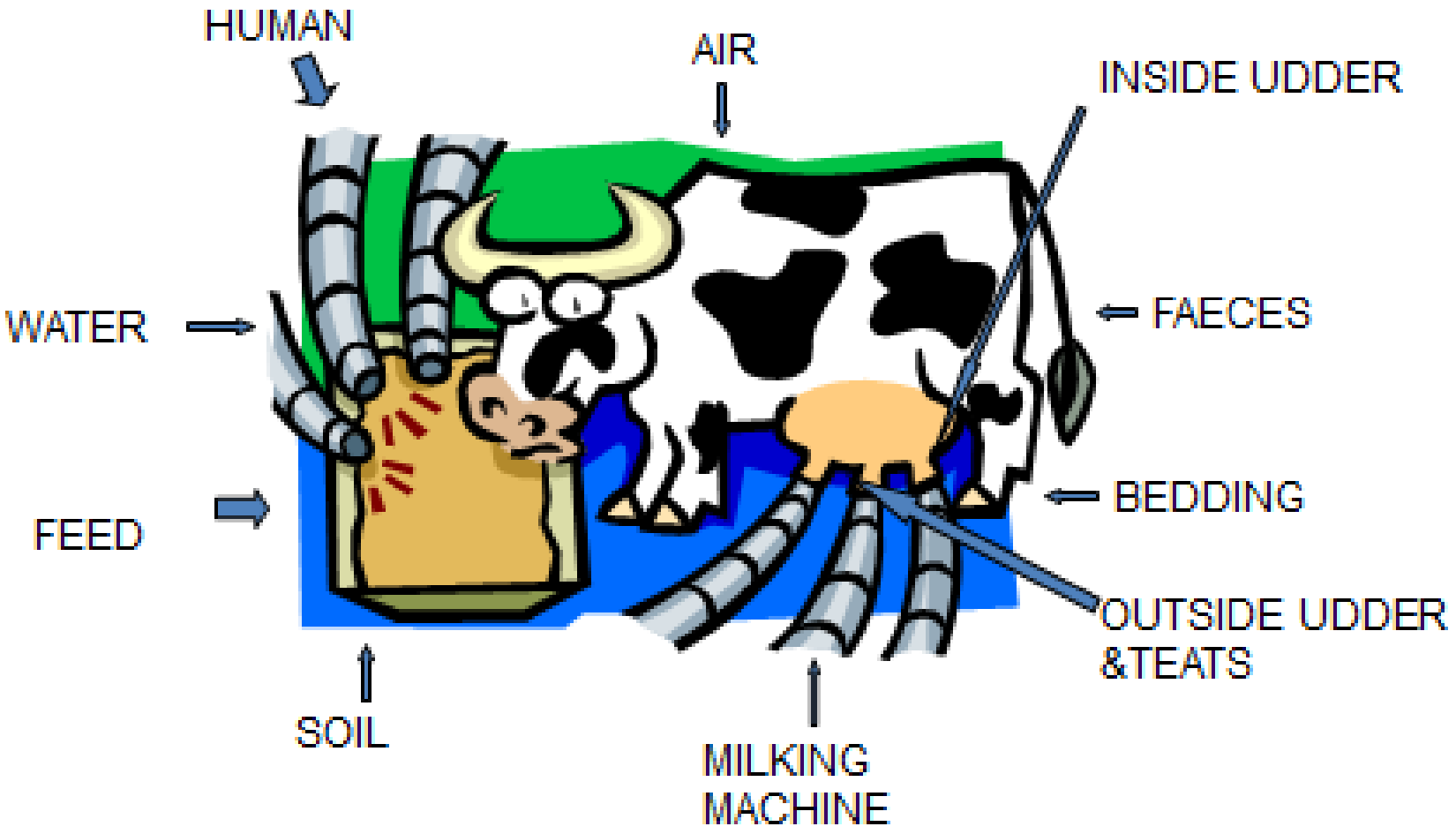
RAW MILK QUALITY: Statistics from 2013 (Maitohygenialiitto ry/ tilastot)

Farming system	Total bacterial counts*	Total somatic cells
Conventional dairy farms	~5300 cfu/ml	~132.000 cells/ml
Organic dairy farms	~5700 cfu/ml	~146.000 cells/m

*only 0.05% exceeded the limit of 10.000 cfu/ml (5.0 log-units)

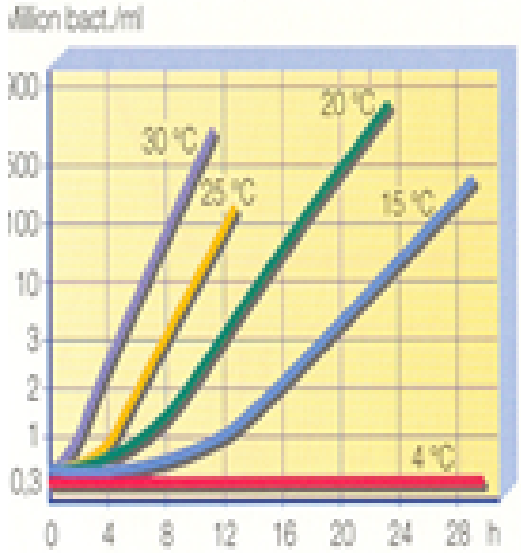
SUMMARY: The microbiological quality of raw milk is excellent in Finland

Sources of microbial milk contamination at the dairy farm (modified from Frank & Hassan, 2002)

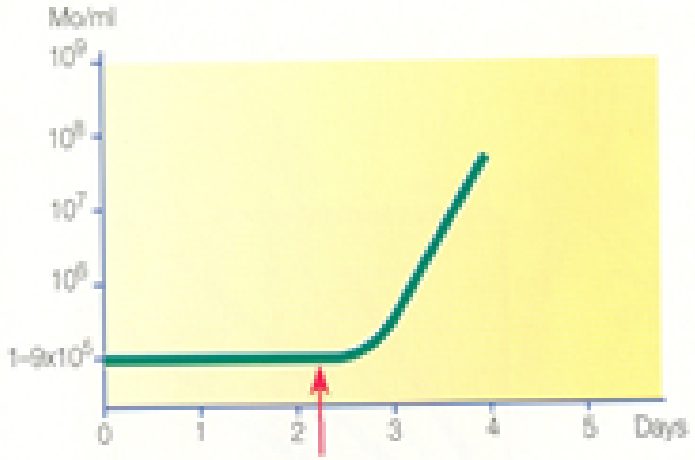


Influence of the temperature on bacterial development in raw milk

Bacterial growth /temperature



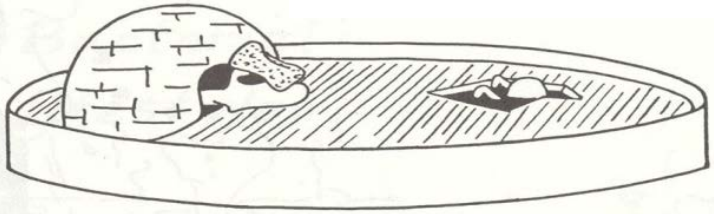
Bacterial growth at 4°C in raw milk



The critical age

(Source: Dairy processing handbook, 2003)

Psychrotrophs



*growth at 5-7°C
*account for less than 10% of the initial raw milk microflora
*comprise numerous genera: Gram-(+): *Bacillus cereus, Clostridium, Staphylococcus…*; Gram-(–): ***Pseudomonas* spp**, *Aeromonas, Serratia, Acinetobacter, Alcaligenes*.....

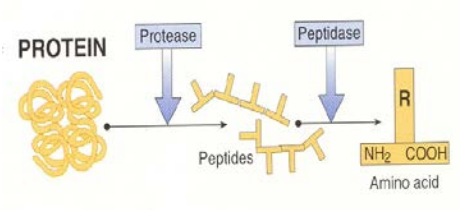
*Some are pathogenic: *Listeria monocytogenes*, *Bacillus cereus*

Psychrotrophs are easily destroyed by low pasteurisation (71.7°C/15 s), but they can cause postpasteurisation contaminations, too.

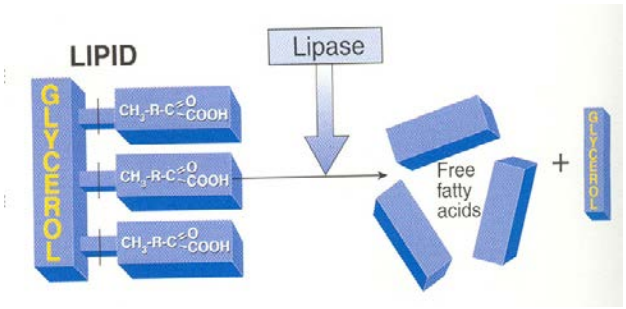
PSYCHROTROPHS SPOILING RAW MILK

Production of extracellular enzymes that are heat-stable

Proteolysis

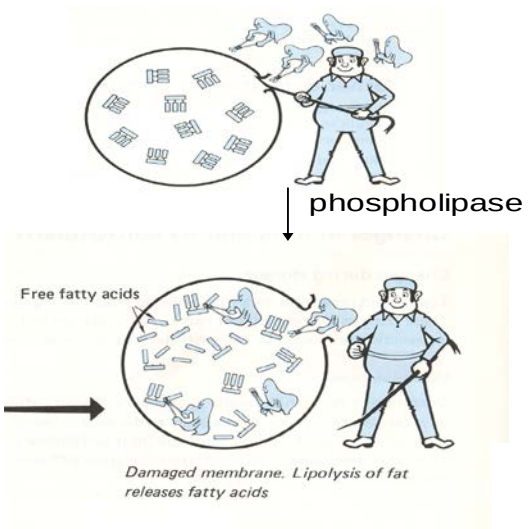


Lipolysis



(Source: Dairy processing Handbook 2003)

Fat globule



Presence of psychrotrophs impacts on the quality of milks and other dairy products:

*milks : gelation of UHT milk, bitter flavours

*cheese production: lower yield, flavour defects (rancidity and soapy)

*butter: rancid and putrid flavours etc…

MICROBIAL QUALITY OF RAW MIK

*EC directive: ***Bacterial levels below 10^5 cfu/ml***

(From the website of BactoScan Tm
”Beating bacteria in milk”)

Analyses of 13 raw milk samples (Farm, Lorry, Silo):
Bacterial isolates were selected for their spoilage features

Results from the **phenotypic characterization**
by API 20 NE and Biolog

*Predominance of *Pseudomonas* spp. spoiling raw milk
*The identity of many isolates remain doubtful or controversial (limitations of the phenotypic identification systems)
*Several isolates attributed to the genera:
Acinetobacter; Stenotrophomonas and *Burkholderia* (former *Pseudomonas*): …by either one or both identification systems (Mic Res 2006,161, 334).

For some isolates, the identity was confirmed by 16S rRNA gene sequencing.

WHAT ARE THESE GENERA?

*confusing taxonomic history

***RESISTANCE TO ANTIBIOTICS!!!!**

” The complete genome of *Stenotrophomonas maltophilia*….reveals an organism heavily shielded by drug resistance determinants ” (Genome Biology 2008)

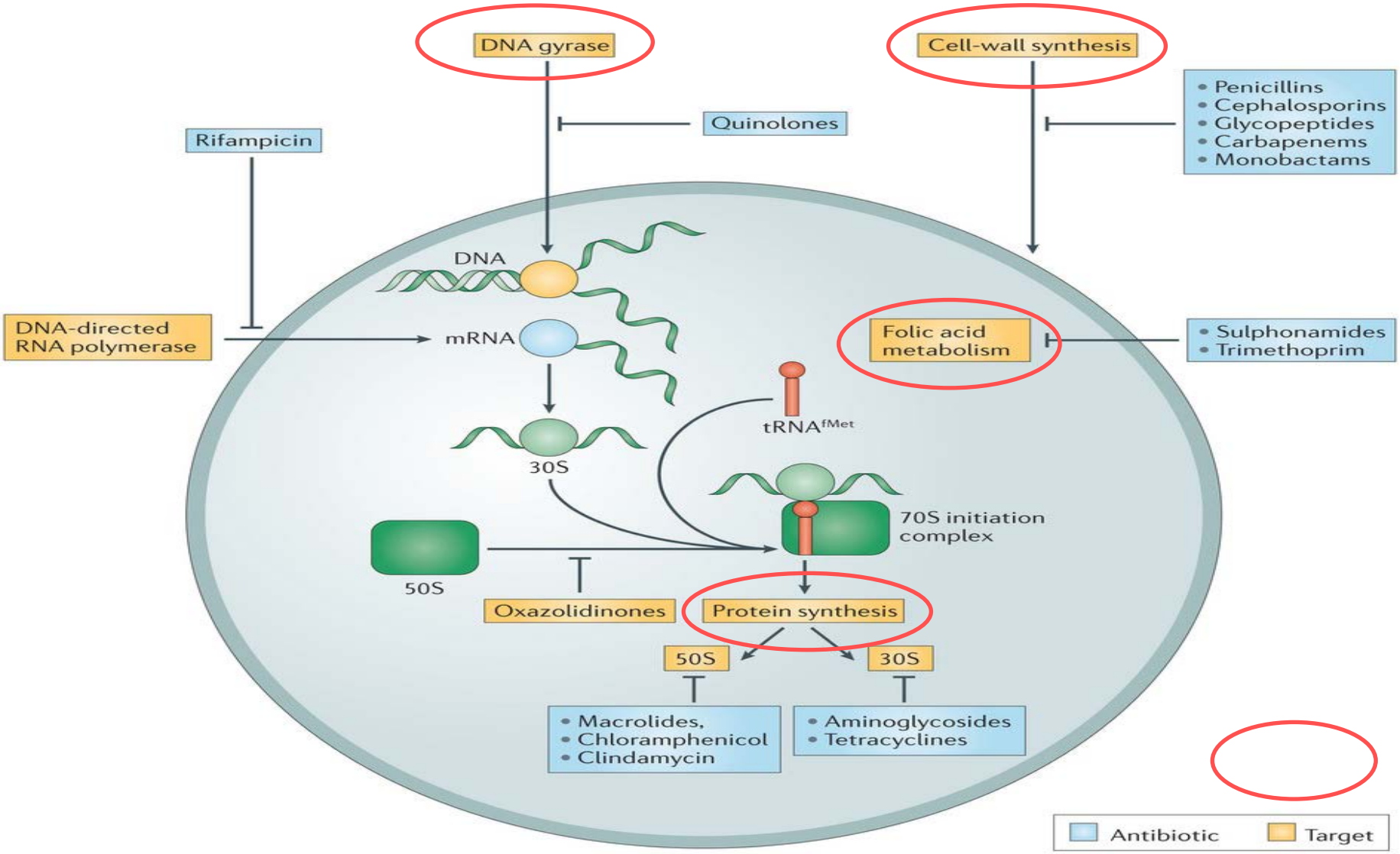
WHAT IS ANTIBIOTIC RESISTANCE (AR) ?

Is a form of drug resistance usually a fraction of the population of a bacterial species are able to survive after exposure to one or more antibiotics.

AR is a serious concern in contemporary medicine, is considered as a **major public health concern**

WHO: ” 30.4.14: this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect every one, of any age, in any country”

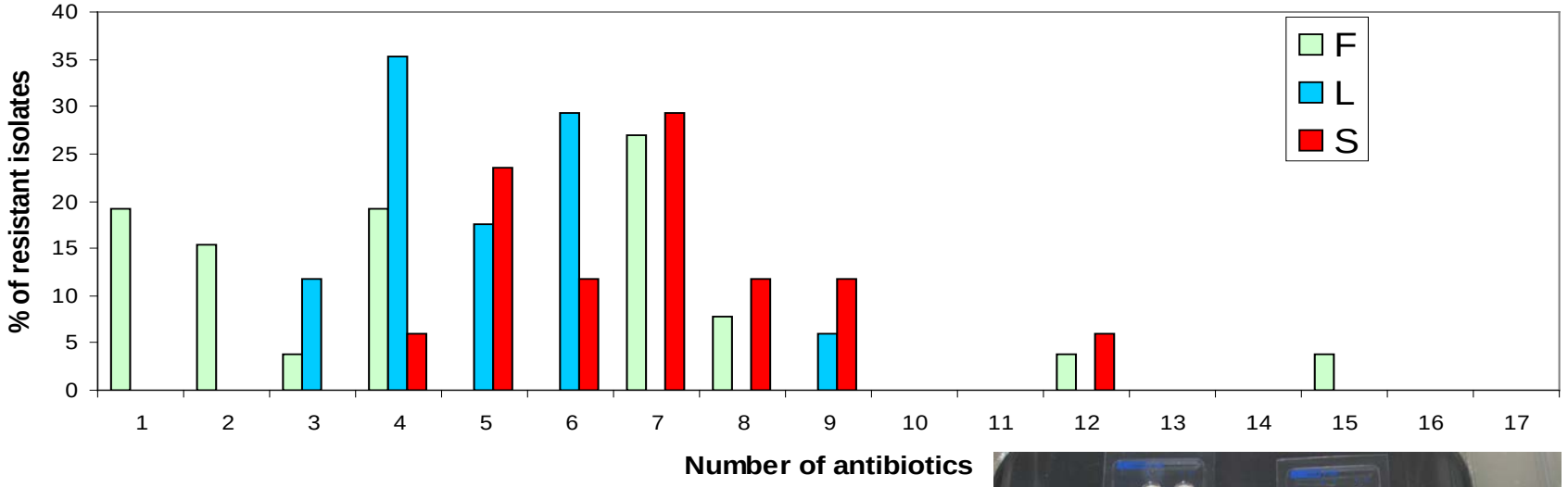
TARGETS AT CELLULAR LEVEL



Nat Rev Drug Dis, 2013 12, 371-387.

ANTIBIOTIC RESISTANCE OF 60 RAW MILK ISOLATES (CONV . SYSTEMS)

Percent of isolates found resistant to a certain number of antibiotics (1 to 17) considering their origin (F, farms; L, lorries;S, silos)



F

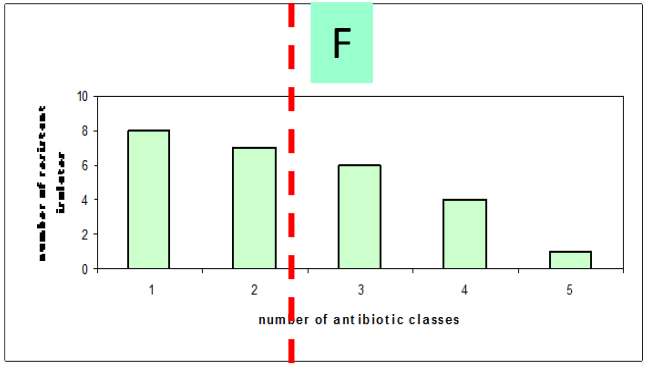
L

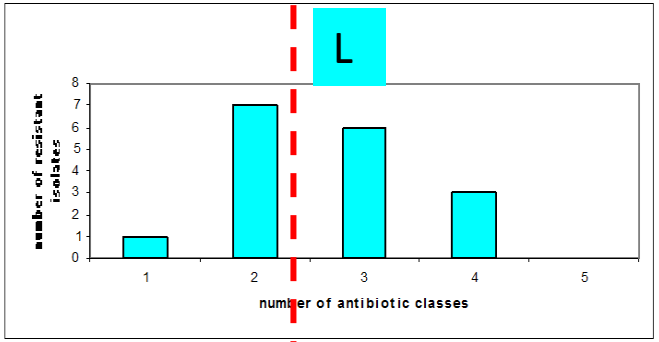
S

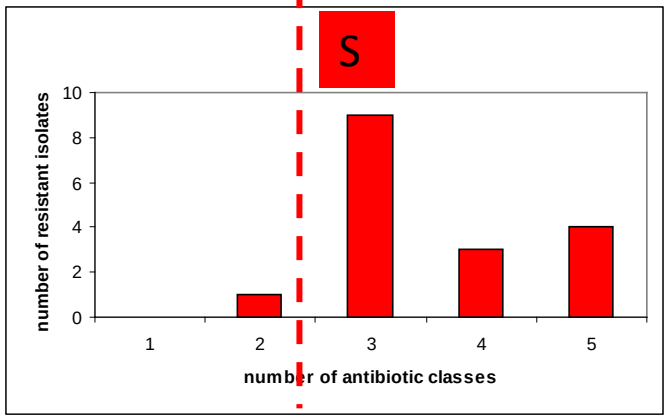
Growth of *Stenotrophomonas maltophilia* Hambi 2659^{T} (left) and the bovine raw milk isolate 101 (right) in the presence of the 17 antibiotics constitutive of the ATB®PSE strip (5 classes)

Picture: O.Gursoy

Breadth of antibiotic resistance (raw milk samples)







F: 42.3% (11/26)

L: 52.9% (9/17)

S: 94.1% (16/17)

} Resistant to at least 3 classes of antibiotics

We observe an increase of this feature along the cold chain of raw milk storage and transportation (Mic. Res. 2007, Vol 162, p.115)

Is the low storage temperature of the raw milk promoting the spread of antibiotic resistance via the psychrotrophs?

Antibiotic resistance (AR) evaluated during cold storage of raw milk

18 raw milk samples (Conv)*:
*6 stored at 4°C (Days 0 & 4)
*6 stored at 6°C (Days 0 & 4)
*6 stored at 4°C (Days 0, 2 & 4)

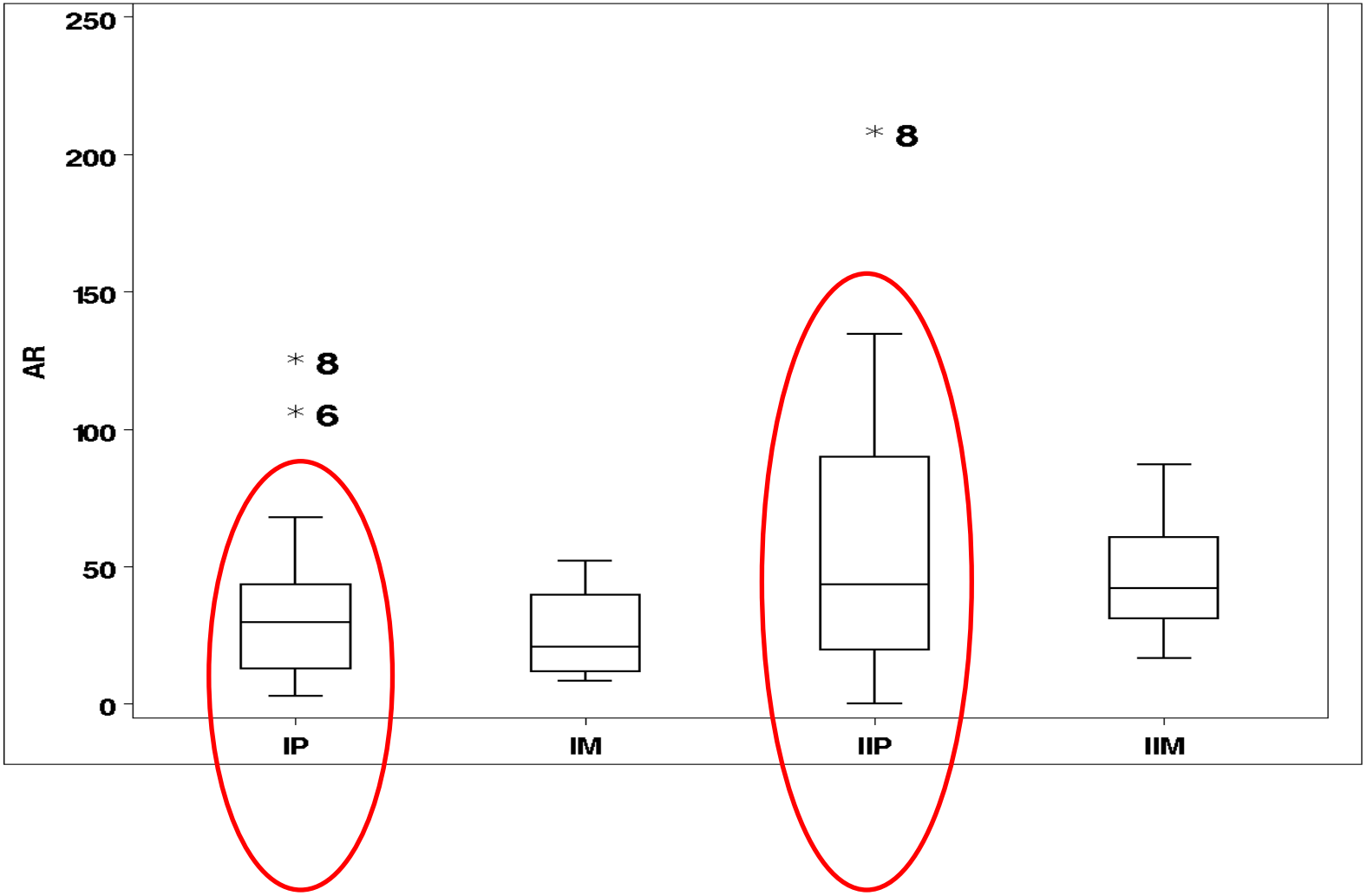
Four antibiotics: G = gentamycin, C = ceftazidim, L= levofloxacin (L), TSU= trimethoprim-sulfamethoxazole

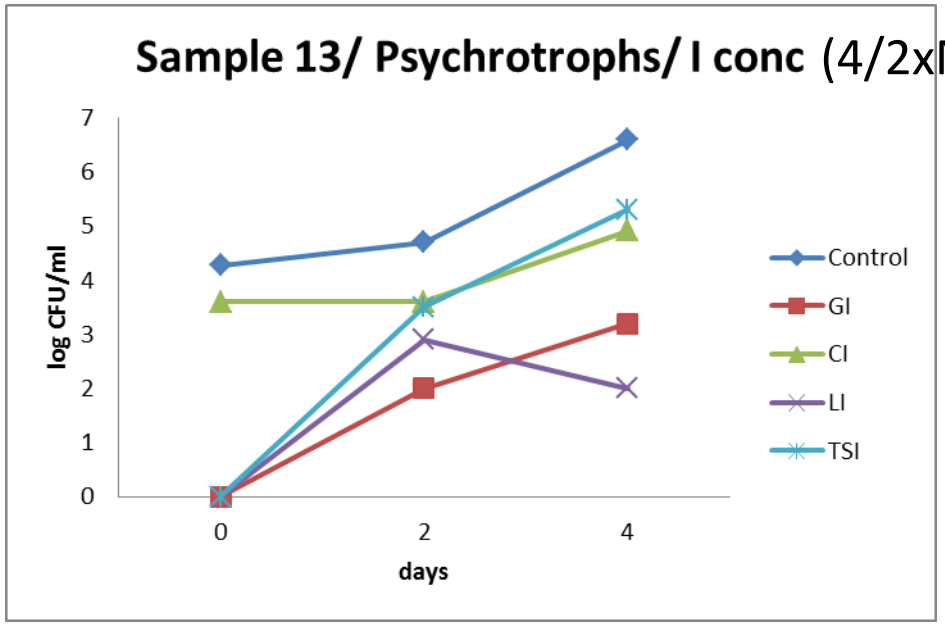
Concentrations in Mueller- Hinton agar corresponded to **MIC**s and to **4xMIC**s for G,C,L and **2xMIC** for TSU, defined for pseudomonads (EUCAST 2000)

*Enumeration of mesophiles (30°C for 3 d) and psychrotrophs (7°C for 10 d)
*Statistics

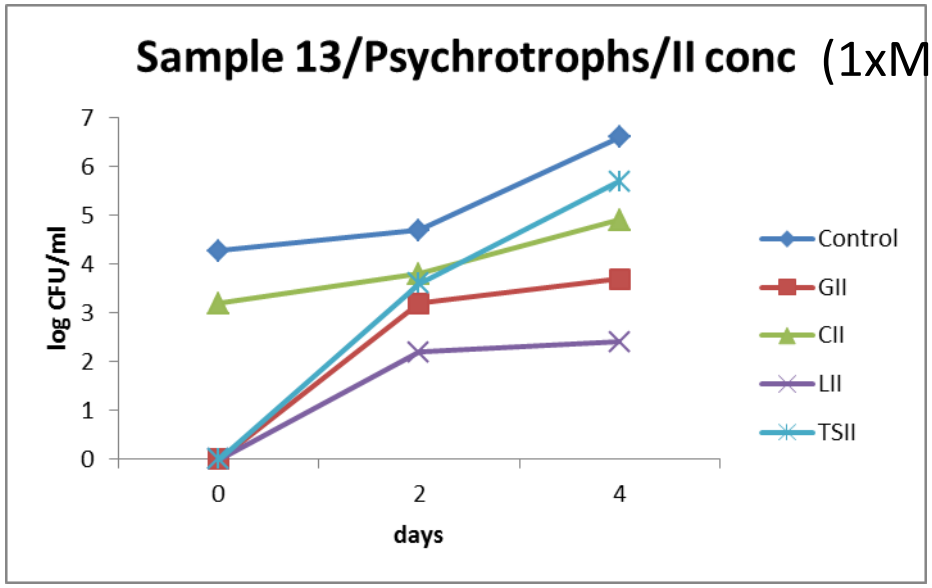
*Initial microbiological quality of the raw milks: excellent (E class) as 17 of 18 samples had less than $5x10^4$ cfu/ml (4.7 log units)

Distribution of the total initial AR percentages for Psychrotrophs (P) and Mesophiles (M)





AR trend of psychrotrophs in one raw milk sample



SUCCESSION OF PSYCHROTROPHIC COMMUNITIES

Mean values of **Rapd** (d=0,2,4) and their corresponding range, for six raw milk samples (13-18, stored at 4°C)

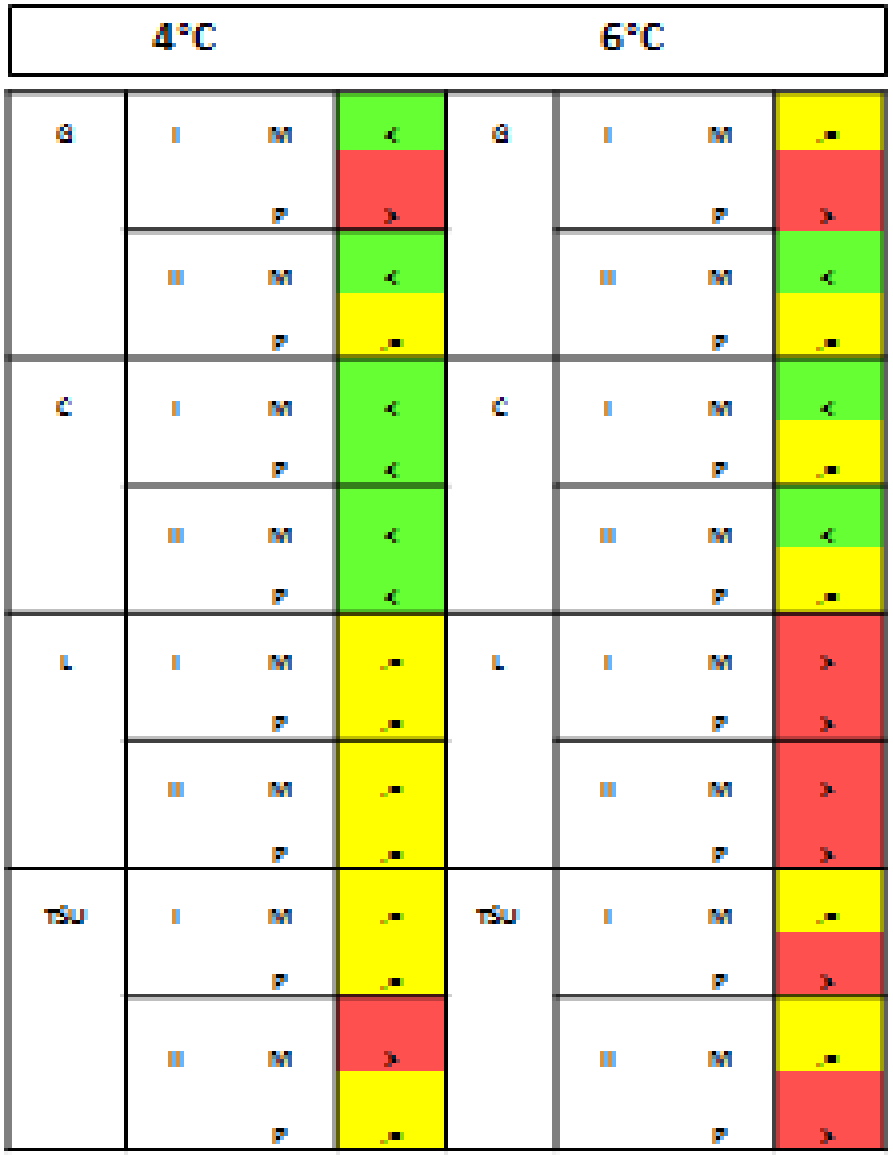
*__Rapd__ is defined for a particular condition X, characterized by a combination of the factors: population type Mesophiles or Psychrotrophs, sampling days 0, 2 or 4, storage T, AB type, AB concentration

	Mesophiles			Psychrotrophs		
Rapd	Rap0	Rap2	Rap4	Rap0	Rap2	Rap4
Mean	0.0788	0.2186	0.02402	0.1387	0.3336	0.0439
Range	0-1.1368	0-5.2363	0-0.5741	0-3.0000	0-9.0333	0-2.000

*AR calculated with Rapd values were always **HIGHER FOR PSYCHROTROPHS**

*Most importantly, all Rapd values were **HIGHEST AT DAY 2**, after 48h storage at 4°C (when total counts were between 4.4 and 5.5 log-units) (ISRN Microbiol, Vol 12, ID 918208)

AR trends evaluated, with the output Rapd, for mesophiles (M) and psychrotrophs (P) present in raw milks stored at 4 or 6 °C during 4 days

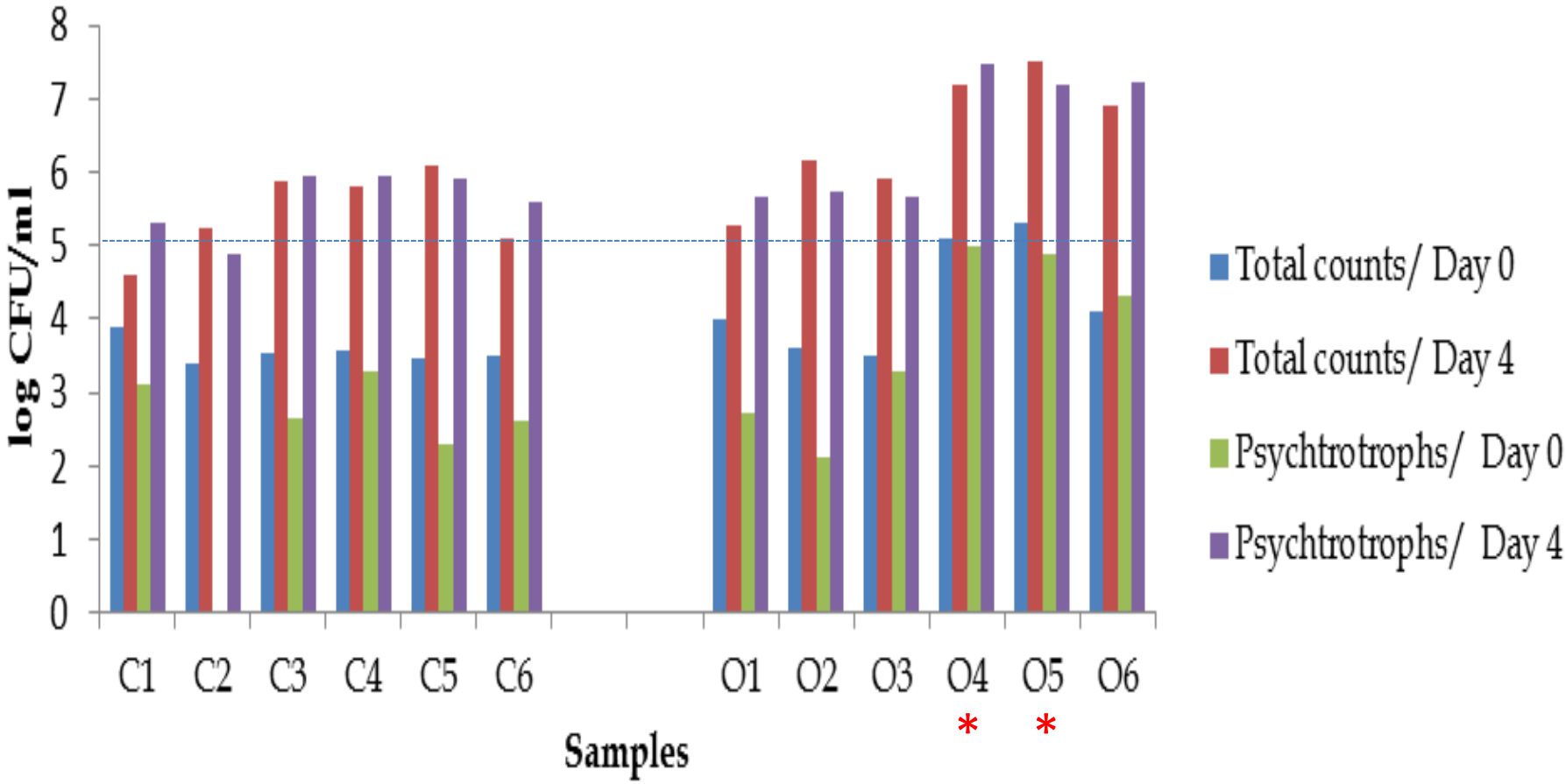


AR at day 4 is superior to AR at day 0 (red), is equal (yellow), is lower (green).

The AR trends suggest that the lowest storage temperature was more appropriate to contain AR at its minimal level.

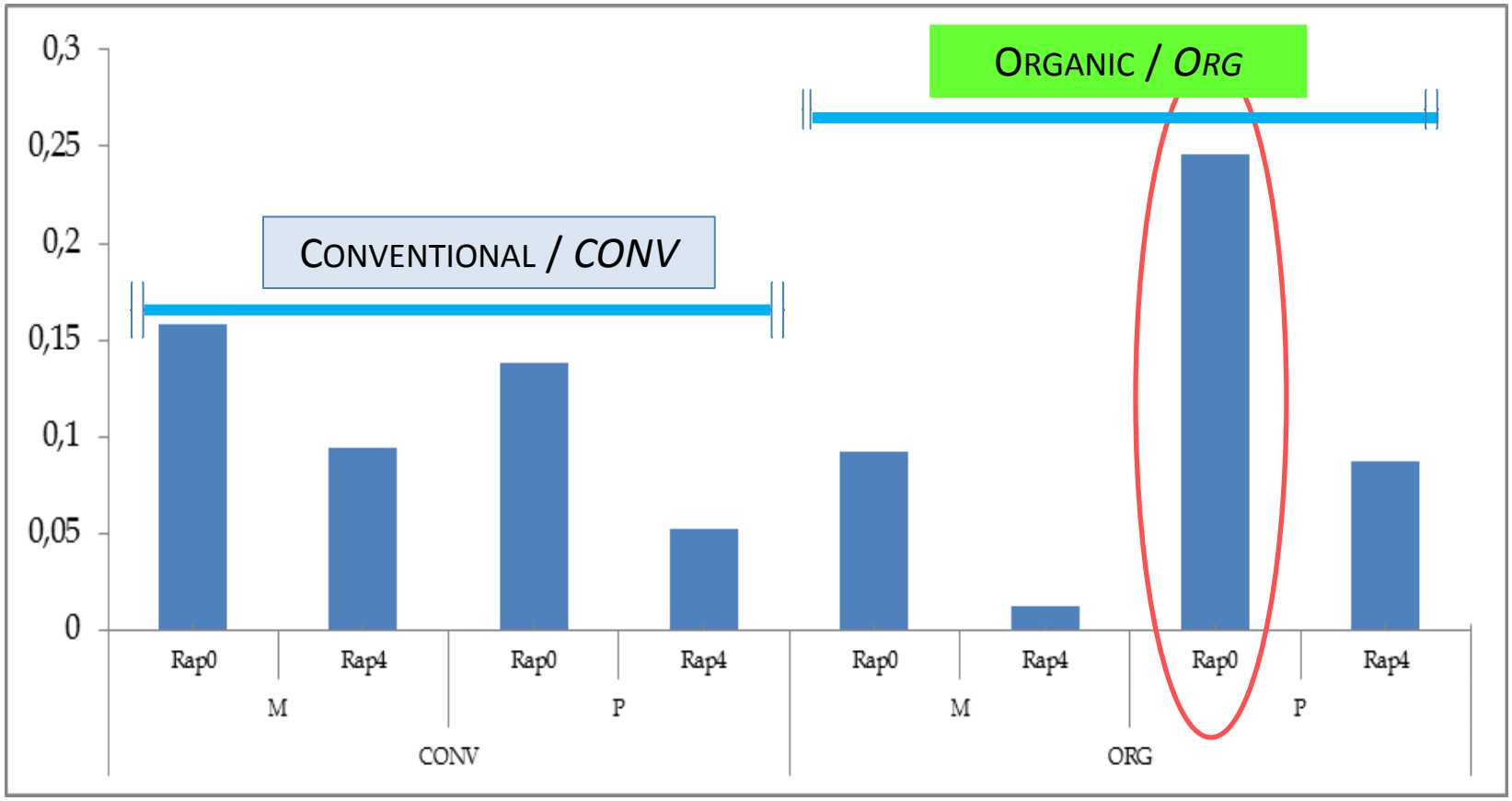
COMPARATIVE STUDY: ORGANIC /CONVENTIONAL FARMING

12 raw milk samples (C1-C6 & O1-O6) cold stored at 4°C for 4 days



*More than 10^5 cfu/ml at Day 0

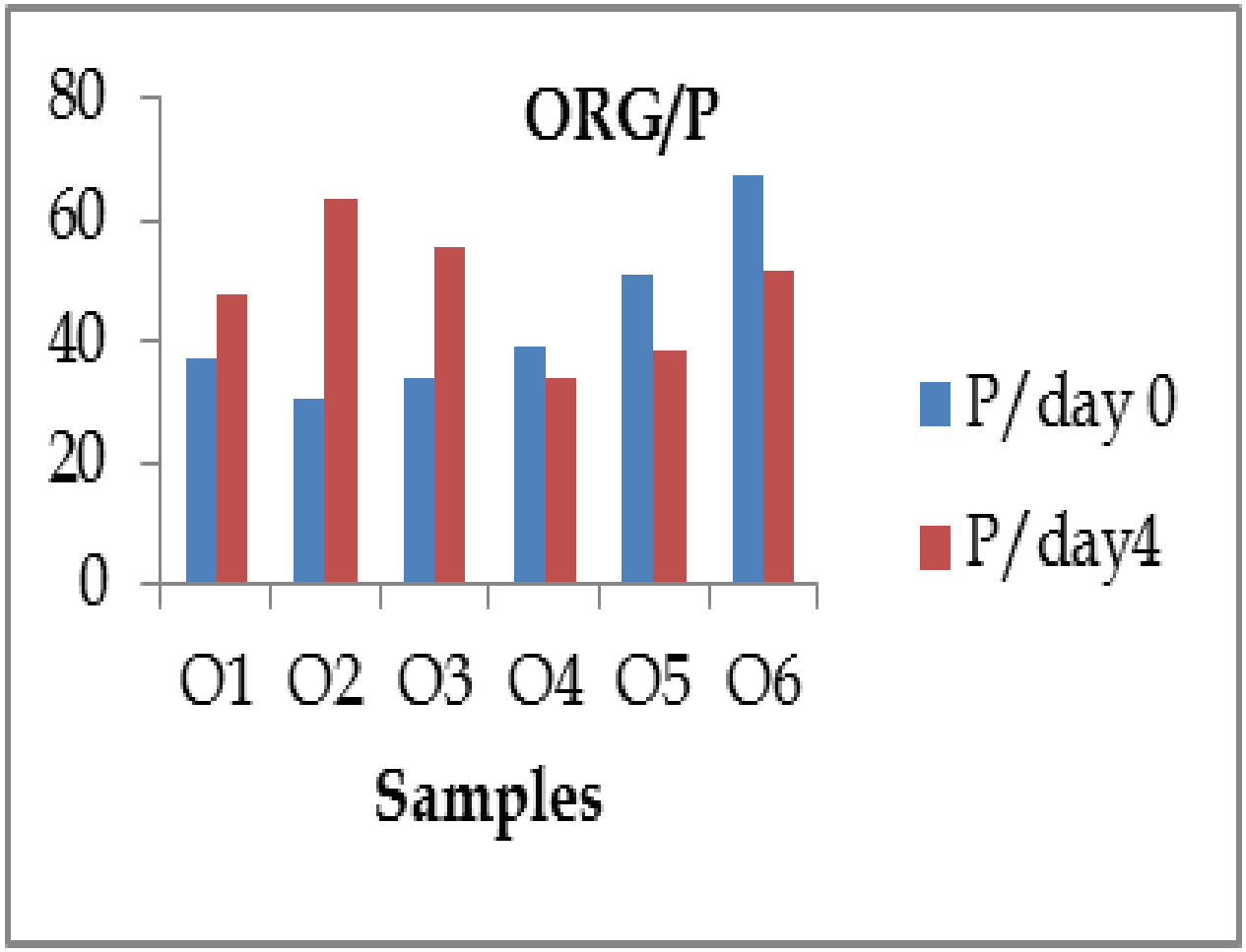
MEAN RAPD VALUES FROM *CONV* AND *ORG* RAW MILK SAMPLES



M = Mesophiles; P = Psychrotrophs

HETEROGENEITY AMONG THE ORGANIC RAW MILK SAMPLES

Ranking of the organic raw milk samples depending on their AR load



COMPARISON OF CONVENTIONAL AND ORGANIC RAW MILK SAMPLES

The symbols =, <, and > are meaning …RAPd4 equalled, or was significantly below or superior to Rapd 0, respectively.

(In Antibiotic Resistant Bacteria: A continuous Challenge in the New Millenium, Intech 2012, 105-124)

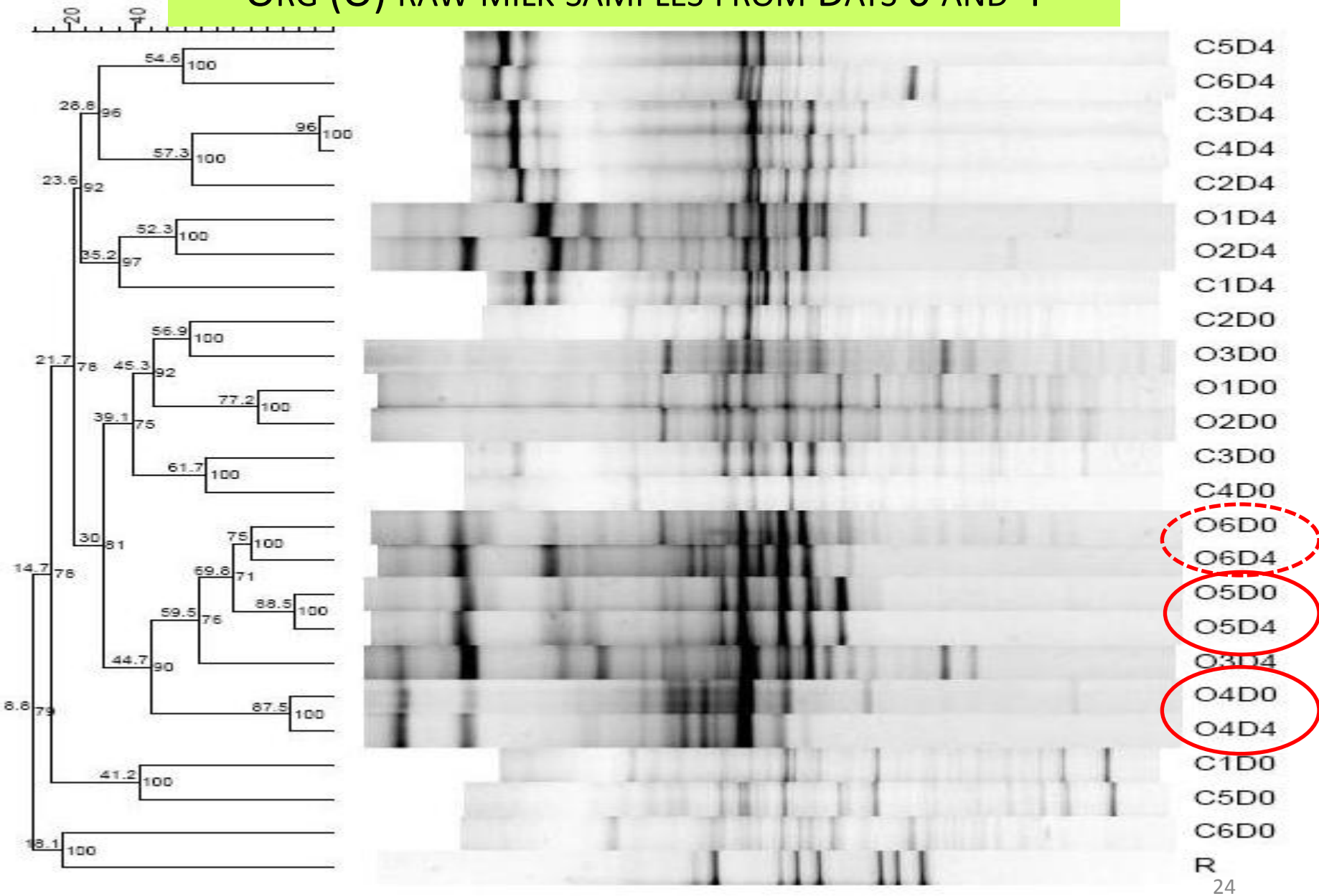
CONVENTIONAL

G	I	M	=
		P	=
	II	M	=
		P	=
C	I	M	<
		P	=
	II	M	<<
		P	=
L	I	M	=
		P	=
	II	M	=
		P	=
TS	I	M	=
		P	>
	II	M	=
		P	>

ORGANIC

G	I	M	>
		P	>>
	II	M	=
		P	=
C	I	M	<<
		P	=
	II	M	<<
		P	=
L	I	M	>>
		P	>
	II	M	=
		P	>>
TS	I	M	>>
		P	>>
	II	M	=
		P	>>

Dendrogram of DGGE profiles from CONV (C) and Org (O) raw milk samples from Days 0 and 4

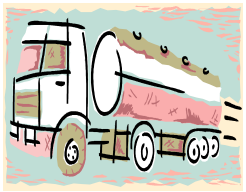


PRODUCTION OF RAW MILK WORLDWIDE

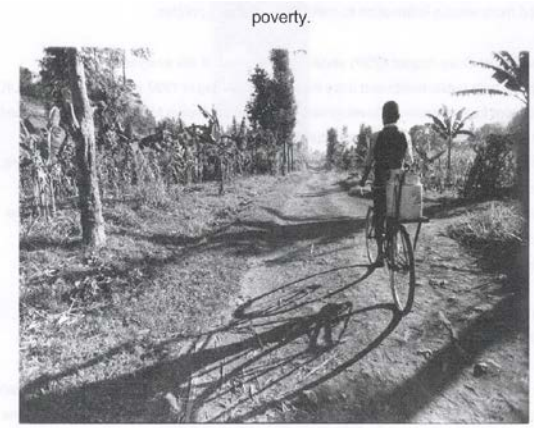
Developped countries

Developing countries :

over 80% of milk consumed are handled by informal market traders, with inadequate regulation (FAO 2009)



EFFECTIVE COLD CHAIN…
(TEMP ≤6°C)



The ubiquitous mobile milk traders in Kenya get about by bicycle, each day travelling 30–50 km and selling 50–120 litres of 'raw' milk, a main food usually drunk as boiled tea.

Internet document

selection of psychrotrophs

…Which spoil raw milk, and which carry antibiotic resistant features…

Some Methods to impede bacterial development in raw and pasteurised milk

Cold chain extension?
… but raises electricity needs… global warming?

Heat treatments/ pasteurisation… promote spore germination

MODIFIED ATMOSPHERE

Addition of CO_2 … sensorial and technological properties are modified…

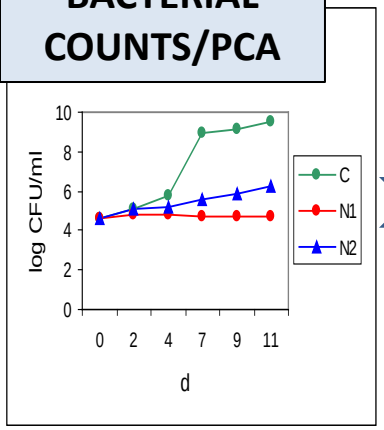
N_2 gas

N_2 (E941) as an inert gas, is allowed in organic food production

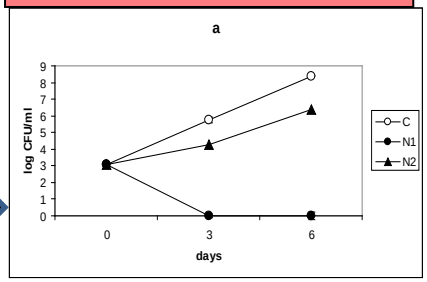
We examined at laboratory and pilot plant scales an ”open system” with continuous control of the headspace atmosphere

NITROGEN GAS (N_2) APPLIED TO RAW MILK

Effect on TOTAL BACTERIAL COUNTS/PCA

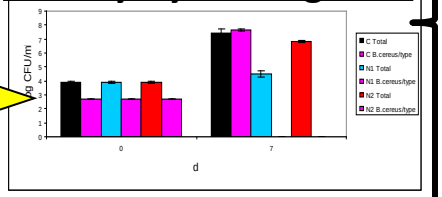


EXCLUSION OF PHOSPHOLIPASES PRODUCERS

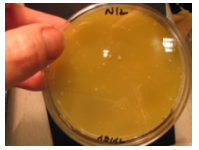


GRAM +

Mannitol Egg Yolk Polymyxin B agar



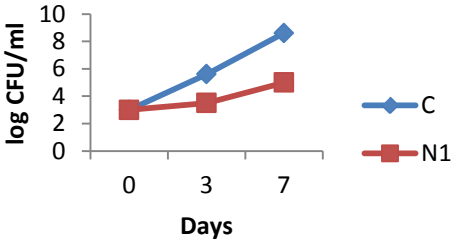
***B. cereus*-type on Control plates**



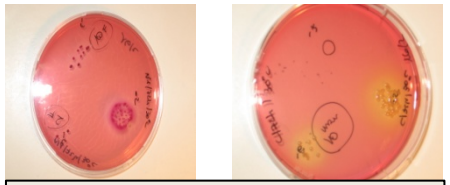
No *B. cereus*-type on N_2 treated milks

GRAM-

Total counts on Mac Conkey Agar



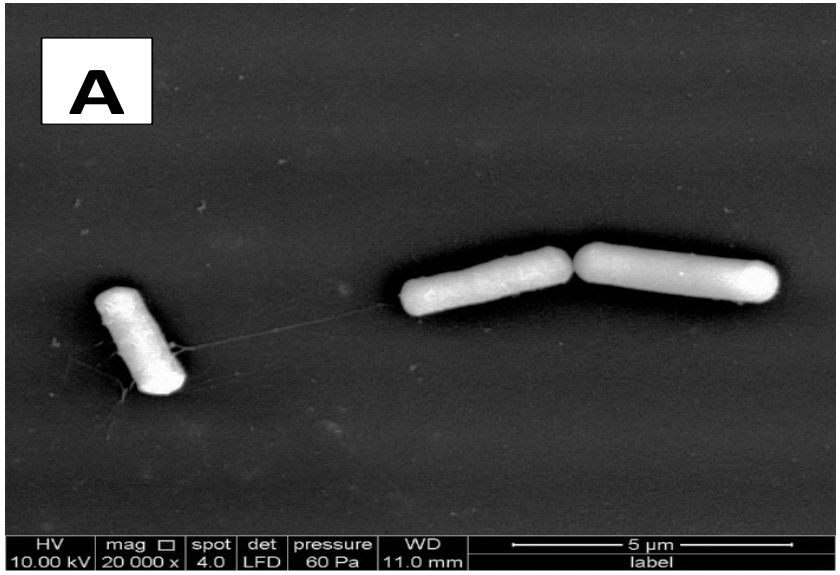
Mac Conkey Agar

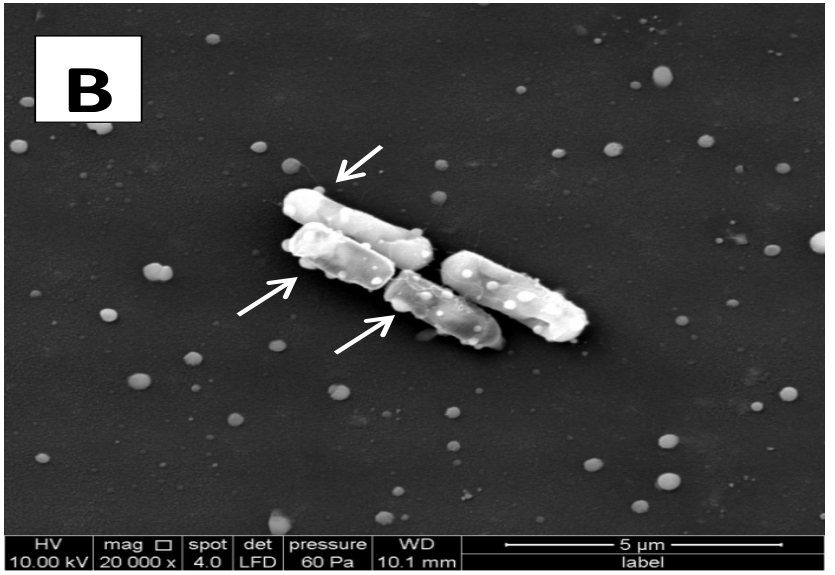


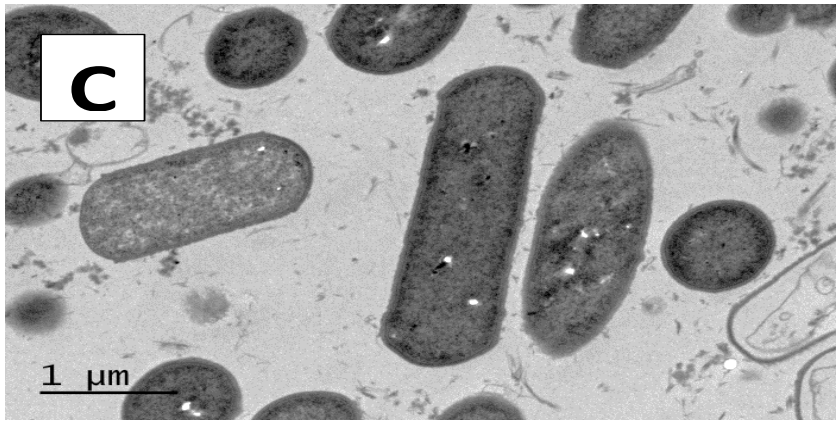
N_2 Control

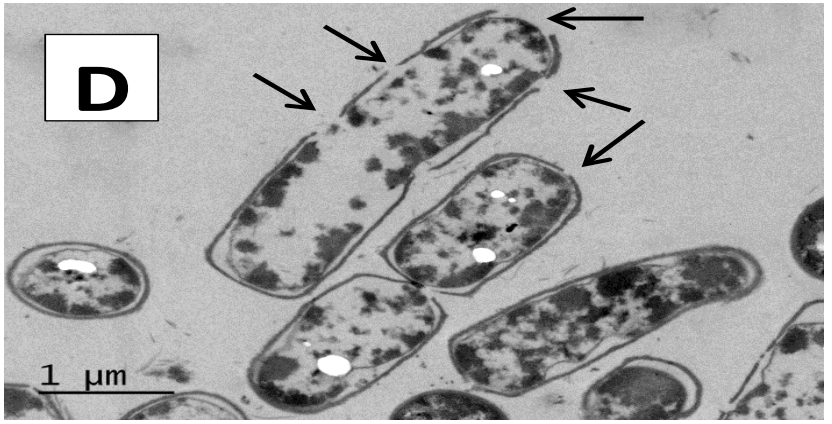
N_2 flushing is preventing the growth of lactose non fermenters like pseudomonads.

First evidence of a bactericidal effect of N_2 gas flushing









(Munsch-Alatossava and Alatossava, submitted)

Conclusions

*Cold storage impacts on AR evaluated from raw milk psychrotrophs

*AR level seems more determined by the ”age of the milk” than by the origin of the milk samples (conventional or organic farming system)

Acknowledgments:

MASTER STUDENTS

Bhawani CHAMLAGAIN

Vilma IKONEN (ROUVINEN)

COLLEAGUES

Jyri REKONEN (samples from conventional farming systems)

Oguz GURSOY, University of Burdur (Turkey)

Tapani ALATOSSAVA (University of Helsinki)

Jean-Pierre GAUCHI, INRA CR de Jouy en Josas (France)

And

Juvan Luomu Oy (samples from organic farming systems)

Thank you for your invitation and for your attention!!