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

Patricia Munsch-Alatossava

Dept. Food and Environmental Sciences
University of Helsinki

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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
* 10,450 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 (Maitohygenialliitto ry/ tilastot)

<table>
<thead>
<tr>
<th>Farming system</th>
<th>Total bacterial counts*</th>
<th>Total somatic cells</th>
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<tbody>
<tr>
<td>Conventional dairy farms</td>
<td>~5300 cfu/ml</td>
<td>~132,000 cells/ml</td>
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<tr>
<td>Organic dairy farms</td>
<td>~5700 cfu/ml</td>
<td>~146,000 cells/ml</td>
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*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

(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.
**Proteolysis**

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
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

- DNA gyrase
- Cell-wall synthesis
  - Penicillins
  - Cephalosporins
  - Glycopeptides
  - Carbapenems
  - Monobactams
- Quinolones
- Folic acid metabolism
  - Sulphonamides
  - Trimethoprim
- Rifampicin
- DNA-directed RNA polymerase
- mRNA
- 30S
- 50S
- Protein synthesis
  - Oxazolidinones
  - Macrolides
  - Chloramphenicol
  - Clindamycin
  - Aminoglycosides
  - Tetracyclines

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

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\(^R\)PSE strip (5 classes)
Breadth of antibiotic resistance (raw milk samples)

F: 42.3% (11/26)
L: 52.9% (9/17)
S: 94.1% (16/17)

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 MICs and to 4xMICs 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 5x10⁴ 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

**Sample 13/ Psychrotrophs/ I conc (4/2xMIC)**

- Control
- GI
- CI
- LI
- TSI

**Sample 13/ Psychrotrophs/ II conc (1xMIC)**

- Control
- GII
- CII
- LII
- TSII

**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

<table>
<thead>
<tr>
<th></th>
<th>Mesophiles</th>
<th>Psychrotrophs</th>
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<tbody>
<tr>
<td>Rapd</td>
<td>Rap0</td>
<td>Rap2</td>
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<tr>
<td>Mean</td>
<td>0.0788</td>
<td><strong>0.2186</strong></td>
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<tr>
<td>Range</td>
<td>0-1.1368</td>
<td>0-5.2363</td>
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*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.

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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
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)

<table>
<thead>
<tr>
<th>CONVENTIONAL</th>
<th>ORGANIC</th>
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**In Antibiotic Resistant Bacteria: A continuous Challenge in the New Millenium, Intech 2012, 105-124**
DENDROGRAM OF DGGE PROFILES FROM CONV (C) AND ORG (O) RAW MILK SAMPLES FROM DAYS 0 AND 4
PRODUCTION OF RAW MILK WORLDWIDE

Developed 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)

selection of psychrotrophs...
...Which spoil raw milk, and which carry antibiotic resistant features...

Internet document
SOME METHODS TO IMPEDE BACTERIAL DEVELOPMENT IN RAW AND PASTEURISED MILK

Heat treatments/pasteurisation... promote spore germination

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

MODIFIED ATMOSPHERE

Addition of CO₂ ... sensorial and technological properties are modified...

N₂ (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**

- **NITROGEN GAS (N₂) APPLIED TO RAW MILK**
  - **EXCLUSION OF PHOSPHOLIPASES PRODUCERS**
  - **Mannitol Egg Yolk Polymyxin B agar**
    - **GRAM +**
      - **B. cereus-type on Control plates**
    - **GRAM -**
      - **No B. cereus-type on N₂ treated milks**
  - **Total counts on Mac Conkey Agar**
  - **N₂ flushing is preventing the growth of lactose non fermenters like pseudomonads.**

- **Effect on TOTAL BACTERIAL COUNTS/PCA**
  - **Days**
  - **log CFU/ml**
  - **C**
  - **N1**
  - **N2**
  - **Days**
  - **log CFU/ml**
  - **C**
  - **N1**
  - **N2**

- **N₂ 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)
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Thank you for your invitation and for your attention!!