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Trends of Antibiotic Resistance (AR) in Mesophilic and Psychrotrophic Bacterial Populations During Cold Storage of Raw Milk, Produced by Organic and Conventional Farming Systems

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1. Introduction

Antibiotic resistant bacteria continually arise and their increasing prevalence constitutes one of the major public health threat. The problem, earlier mainly confined to hospitals, nowadays encircles the globe (Davies & Davies, 2010; Levy, 2002; Marshall et al., 2009). Perceived once as a consequence of use, overuse and misuse of antibiotics to prevent or treat diseases, as growth promotants for food animals, or as pesticides for agriculture, more explanations for high AR levels in bacteria were recently brought. The picture became darker when came evidences that environmental microbiota present in antibiotic free environments showed to possess an as enormous and diverse number of AR genes as present in pathogenic microbiota (Aminov, 2009). Further evidences point to micro-organisms associated with food, animals, and water as the main sources for resistance genes; commensals among them food commensals are also considered as a reservoir of AR (Knezevic & Petrovic, 2008; Straley et al., 2006), in fact according to Marshall et al. (2009) a rather underappreciated reservoir of AR. In developed countries, several parameters define a raw milk of "good quality" when it is absent of drug or antibiotics residues, when the legal limit of somatic cells per millilitre of milk is below $4x10^5$ /mL (excessive values may be indicating the presence of mastitis in the cow herd), when the bacteriological acceptance level is satisfied. In the later, the sanitation of raw milk is ensured by the determination of the standard plate count (Chambers, 2002) that aims to enumerate aerobic "total bacteria" present in milk; grade A or 1 (acceptable for industrial use) is attributed to milk that contains less than $1x10^5$ CFU/ml, determined on agar plates after 2 days incubation at 32°C, or 3 days at 30°C. After milking, numerous contamination sources raise the bacterial load

along the cold chain of raw milk storage and transportation (Chambers, 2002; Cousin, 1982). The cold storage that aims to preserve food or milk from excessive bacterial development, however also selects bacterial types which have perfectly adapted to low temperatures : psychrotrophic bacteria, able to grow below 7°C, present in raw milk are well known for their spoilage features (production of various heat-stable exoenzymes) which affect raw or processed dairy products with significant economic impact. Mainly, out of some exceptions like the human pathogens (toxin producer of *B. cereus* species, or *Listeria* spp: Gray et al., 2006; Schoeni & Lee Wong, 2005), most psychrotrophic bacteria associated to raw milk of which many are Gram-negative, are generally considered as benign.

Foodstuffs are produced by either conventional (CP) or organic (OP) systems. Consumer demands for organic products generally perceived as more safe, is growing in Europe and the United States, offering increased business opportunities and wealth for rural regions (European Commission, 2008; Jacob et al., 2008). The organic food chain supply is guaranteed at the base first by producers which must adhere to strict rules: organic milk is defined by the European Commission as "milk that comes from cows, sheep and goats living in a welfare-oriented animal husbandry: outdoors in summer with access to pasture and indoors in winter when the climate is rough, with organic forage and enough space for regular exercise" (European Commission, 2008). Several principles are underlying organic production such as minimisation of the use of non-renewable resources and off-farms inputs, recycling of wastes and by-products of plant and animal origin as input in plant and livestock production, the feeding of livestock with organic feed (produced mainly at the farm), synthesized allopathic veterinary medicinal products, like antibiotics may be used with restriction on courses of treatment and withdrawal period (European Council Regulation, 2007); organic dairy cattle are treated for mastitis with the same antimicrobials as dairy cows from conventional systems. In Finland, at least 50% of the feed has to be produced by the farm; each cow can only be treated 3 times a year for independent diseases and the time for milk delivery acceptance to dairies is twice as long as for normal systems (Finnish Food Safety Authority, 2008); on a total of 2.2×10^9 L of milk delivered to dairies about 1.3% was produced by organic farming systems (Information Centre of the Ministry of Agriculture and Forestry, 2009). The use of ABs in Finland for cattle was surveyed by Thompson et al. (2008): for acute mastitis, parenteral treatments are based on benzyl penicillins (83%) and fluoroquinolones (11%); ampicillin combined with cloxacillin (36%), or cephalexin combined with streptomycin (26%) were intramammarily administered. Finland, together with Norway and Sweden, have lower ABs usage practices compared to seven other European countries (Grave et al., 2010). Considering the bacteriological quality of the milk, as well as the level of antimicrobial residues (Finnish association for milk hygiene, 2008), altogether the quality of Finnish raw milk is excellent.

While characterizing some raw milk gram-negative psychrotrophs, it was observed that besides having spoilage features (Munsch-Alatossava & Alatossava 2006), these bacteria also carried antibiotic multiresistant features: moreover, the study suggested that the AR load was higher for isolates that apparently spent a longer time in cold storage (Munsch-Alatossava & Alatossava 2007); another study, that considered bacterial raw milk psychrotrophs selected for their spoilage features, compared the AR levels of 79 bacterial isolates originating from CP (6) or OP (9) milk samples. With exception of gentamicin for which similar percentages of AR were recorded for CP and OP samples, we observed a

lower prevalence of AR for OP samples; resistance levels to trimethoprim-sulfamethoxazole were 25 and 14% for CP and OP samples respectively, and resistance percentages were higher for ceftazidim and ciprofloxacin (a quinolone) for CP-originating isolates (Munsch-Alatossava, Xheng, Alatossava, unpublished data). To further answer to the question on whether AR levels may be different/lower for isolates retrieved from OP compared to CP milk samples, to follow the respective trends for mesophilic and psychrotrophic populations over time during cold storage (4 days at 4°C), the present study was undertaken: the AR to four ABs (gentamicin, ceftazidim, levofloxacin, and trimethoprim-sulfamethoxazole, representatives of 4 different classes) was evaluated for mesophilic and psychrotrophic bacteria for 12 raw milk samples (6 for each farming system); changes at the bacterial communities level during cold storage were investigated by DGGE.

2. Materials and methods

2.1 Cold storage of raw milk samples

Representative bovine raw milk samples of lorry tanks were collected into sterile bottles; samples were kept on ice until arrival at Helsinki University, at which time 100 ml were added to sterile 250 ml-bottles. Six bottles were placed on a multi-place magnetic stirrer (Variomag) and partially immersed in a refrigerated water bath (MGW Lauda MS/2) which allowed, with help of an immersion thermostat, a constant temperature to be maintained (modified from Munsch-Alatossava et al., 2010). The raw milk samples were continuously mixed at 220 rpm and kept at 4± 0.1 °C for 4 days.

2.2 Antibiotic resistance

The experimental procedures followed the EUCAST guidelines (2000). The microbiological analyses were performed immediately after milk samples arrived; all bacterial counts were determined from duplicate or triplicate agar cultures at day 0 (shortly after reception of the samples) and day 4 (after cold storage); 500 µl or raw milk were serially diluted in saline solution (0.85 % NaCl); 50µl of the diluted samples were spread on Mueller-Hinton (Lab M) agar plates. Four antimicrobial agents [gentamicin (Aminoglycosides), ceftazidim (β-lactams, Cephems), levofloxacin (Quinolones) and trimethoprim-sulfamethoxazole (at a ratio of 1/19, a Folate pathway inhibitor) (Sigma)] were added to agar, according to the EUCAST guidelines (EUCAST, 2000). The ABs solutions were freshly prepared by dissolving the powders in following solvents: water for G (gentamicin), 0.1M phosphate buffer (pH7) for C (ceftazidim), 0.1M NaOH for L (levofloxacin), 0.1M lactic acid for T (trimethoprim), and 95% ethanol for S (sulfamethoxazole) (EUCAST, 2000). With exception of S, all AB solutions were filter sterilized prior to the addition to adequately cooled agar. The AB concentrations were 16 mg/L for GI, 4 mg/L for GII, 32 mg/L for CI, 8 mg/L for CII, 8 mg/L for LI, 2 mg/L for LII, 8 mg/L trimethoprim with 152 mg/L sulfamethoxazole for TSI, and 4 mg/L trimethoprim with 76 mg/L sulfamethoxazole for TSII, which correspond to the MICs (GII, CII, LII and TSII) to 4-fold the MIC (GI, CI, LI), or to 2-fold the MIC (TSI) as indicated by EUCAST for pseudomonads. Agar plates were stored overnight at 4°C, and protected from light. Following the analyses, the plates were incubated for 2-3 days at 30°C, or for 10 days at 7°C to enumerate the "total" bacteria (mesophiles) and psychrotrophs, respectively.

2.3 Statistical analysis

2.3.1 Judicious remarks about ANOVA (ANalysis Of VAriance)

Usual analysis of variance is well known in the field of research and laboratories as an efficient statistical method enabling to analyse results following experimental designs, and to test for significant differences between means. Concerning milk and its microflora, also ANOVA was used (Freitas et al. 2009; Ma et al. 2003). Thanks to the Fisher-Snedecor and Student tests, ANOVA enables to detect factors, to highlight interactions of the considered factors, which both significantly impact on the response (continuous) of the studied phenomenon. However, ANOVA is not considered as robust as it is susceptible to variations of the assumptions on which this method is grounded; more precisely, the statistical tests´ validity of ANOVA are "sensitive" to these variations. The validity is relying on three fundamental restrictive assumptions: A) Distribution of the residuals is normal; B) Variance of errors is constant; C) The data does not contain outliers. If one of these hypotheses is strongly violated, conclusions about significant level of the effects of the factors may be questionable or erroneous. In practice for a particular study, the hypothesis A is very difficult to be proved due to usual low amount of repetitions for a certain treatment (a combination of factors set at a certain level); in addition, if this assumption is not respected the impact on the Student test result is rather low, contrarily to the Fisher Snedecor test; however, this hypothesis has been so often checked by numerous experimental studies, that one may assume it is approximately often respected.

The hypothesis B is easily checked on the graphical analyses of "residuals"; if the hypothesis B is not respected, one common way to proceed even though not optimal consists in stabilizing variation by considering the logarithmic values of the results of the response. Also from the observation of the usual ANOVA graphics (given by standard statistics software) the hypothesis C can be checked. Mathematically it can be demonstrated that statistical tests are hampered by the presence of many outliers which may lead to erroneous conclusions. Moreover in the presence of orthogonal or almost orthogonal experimental designs, the outliers promote high levels (for example triple) of interactions of no meaning; typically one interaction AxBxC between three factors A, B, C may be declared significant, even though in the ANOVA model none of the three main effects of the three factors appears. How to overcome the harmful impact of the outliers on the significance of the ANOVA model? The elimination of extreme results due to outliers constitutes one solution, however in the presence of a limited amount of repetitions in practice this option is not applicable. Consequently it is difficult to validate the hypothesis C. To overcome this problem, no ideal method exists. Nevertheless, to compare several samples one alternative consists in the use of non parametric statistical tests like the Kruskal-Wallis test (Conover 1980) for example, which considers the ranks of the results of the response. For example, for the 6 following results of bacterial counts (expressed as CFU/ml) 0, $1.9x10^4$, $1.5x10^5$, $2.0x10^5$, $2.4x10^5$ and $1,5x10^9$, the extreme values 0 and $1,5x10^9$ may be badly estimated by the ANOVA model, and will generate excessive residuals. With a non parametric statistical approach, the values will be substituted for ranks, here 6, 5, 4, 3, 2, and 1.

2.3.2 RAPD definition

The data analysed in this study are bacterial counts enumerated on Petri dishes, characterized by a rather high variability, which impacted on hypothesis C, and which did

not permit the straightforward use of a reliable ANOVA model. Consequently, bacterial counts were transformed into ratios, which were replaced then by ranks according to the prerequisite of the Kruskal-Wallis test, before performing the classical ANOVA. What is meant by ratio? Due to the natural microbiological variability, we considered the CFU (colony forming units) on a Petri dish (in the presence of one AB) not as an absolute value but as a relative value compared to a control plate, which implicated the introduction of a ratio. The ratio here referred to as RAPD was defined for a particular treatment X. RAPD corresponds to the ratio of the amount of bacterial colonies (as CFU/ml) enumerated under this treatment X divided by the number of bacteria enumerated on the corresponding control plates (in the absence of the AB). The treatment X was characterized itself by a combination of factors, like the sample type (milk from CP or OP systems), the population type whether psychrotrophs (P) or mesophiles (M), a sampling day D (D= 0 or 4, that will lead to RAP0 and RAP4 respectively), an AB type, and an AB concentration.

Thus RAPD constitues another way to quantify AR prevalence, while the classical quantification of AR prevalence is generally defined as the percentage of resistant bacteria considering the corresponding "total" bacteria enumerated.

2.3.3 Experimental design

The experimental design was based on the following four fixed factors: the antibiotic (AB) type (whether G, C, L or TS, as detailed above), the concentration of the AB (Dose) which corresponded to a higher level (I) or a lower level (II), the storage time of the milk (day) whether 0 (initial counts) or 4 (after 4 days cold storage), and finally the milk sample noted (ECH) which accounted for the six distinct lorry tanks samples (whether from CP or OP systems). The factor ECH was also introduced as a fixed factor in order to identify an eventual "milk collecting effect". Each treatment corresponded to a defined condition resultant from one modality of every factor (AB, Dose, ECH).

2.3.4 Refinement of ANOVA

Once the ANOVA model has been established, significant factors are identified. If one significant factor presents only 2 modalities, the interpretation is clear: a change of the modality impacts significantly on the level of the response. But if the significant factor presents more than two modalities, the interpretation is not straightforward. Further analyses of the microbiological data are requested, which are not often performed according to the microbiological literature. After the ANOVA table is established, for factors with more than two modalities, pairwise or multiple comparisons of the means of the response associated to the modalities of the factors are requested. On a statistical point of view, as this latter is the most rigorous approach, it was employed in this study. Among the available methods for multiple comparisons of means was chosen the REGW test (Einot & Gabriel, 1975; Ryan, 1959, 1960; Welsch, 1977) that is powerful and particularly adapted for our type of data. The method is available on the SAT/STAT version 8.1 software (SAS Institute, NC, USA).

2.3.5 Use of a non parametric statistical test

One major aim of this study was to compare the trend of RAPD between the two sampling days (Day 0 and Day 4), for a certain treatment. Considering the non normal distribution of

the 12 values of RAP0 and RAP4, the mean comparison with a Student test was not possible. Therefore, the analyses were pursued with the use of the non parametric Wilcoxon test (as detailed in page 215 by Conover 1980), implemented in the NPAR1WAY procedure of the SAS/STAT statistical software. This test is also based on ranks: eight treatments (AB=G at dose I, AB=G at dose II.....AB=TS at dose II) were examined for each bacterial type; for both conventional and organic milk types, altogether 32 conditions were considered.

2.4 DGGE (Denaturing Gradient Gel Electrophoresis) analyses

Bacterial DNA was extracted with PathoProof™ mastitis PCR assay kit (Finnzymes, Finland). 16S rDNA sequences were amplified by nested-PCR. Firstly, a 700-bp fragment that comprises the V3 region of bacterial 16S rDNA was amplified, and served as template for the 2nd PCR reaction which yielded PCR products of about 200 bp, as described by Ogier et al. (2002). These primers flank the V3 region (that corresponds for *E.coli* to positions 436-499) which shows variability between different species. DGGE analyses were performed with the BioRad DCode ™ Universal Mutation Detection System (BioRad, USA). The samples were electrophoresed with a denaturing gradient of 35-70% urea and formamide at 70V for 21h. Gels were stained with SYBR Gold (Invitrogen, USA) and photographed on a UV transillumination table (UVItec Ltd, UK). The images were analysed with Gel Compar®II (version 5.1, Applied Maths, Belgium). The similarity between samples was calculated with Pearson´s correlation coefficient and UPGMA (Unweighted pair-group method using arithmetic averages) was used as a clustering method. The maximum parsimony cluster analyses were performed with the boostrap value of 1000. The data is presented as a dendrogram of DGGE profiles from conventional (C1 to C6) and organic (O1 to O6) raw milk samples at days 0 and 4.

3. Results

3.1 Bacterial counts and percentage of psychrotrophs

Initial "total" bacterial counts, determined for the raw milk samples (C1 to C6, O1, O2, O3 and O6), were comprised between 3.4 and 4.12 log-units, indicating that the raw milks were of excellent quality (Fig. 1).

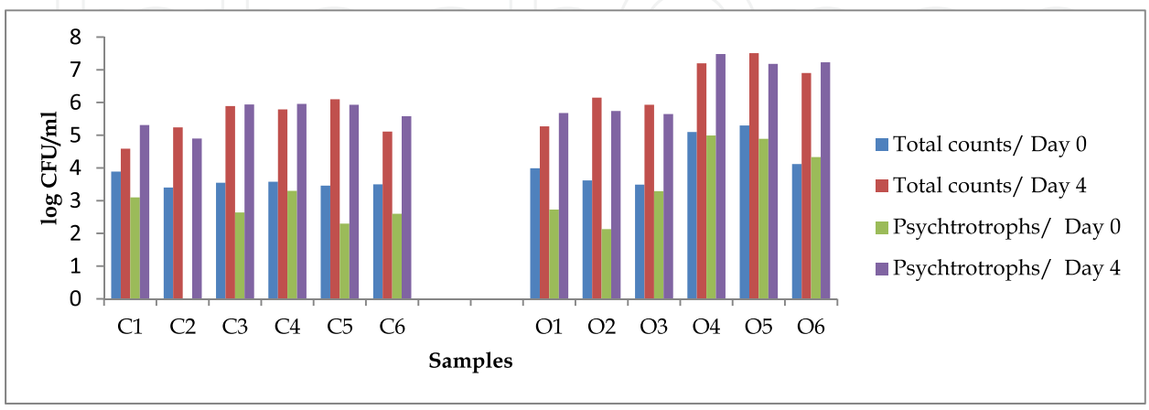


Fig. 1. Bacterial counts on Mueller Hinton agar plates expressed in log CFU/ml, determined for conventional (C1 to C6) and organic (O1 and O6) raw milk samples.

For samples O4 and O5, the mesophilic bacterial counts were 5.1 and 5.3 log-units respectively, suggesting that these milks were longer cold stored prior to the analyses of these samples. At day 4, total counts exceeded 10^5 CFU/ml for all considered samples, to the exception of C1 for which the growth was only of 0.7 log-unit. With exceptions of C4, O3, O4, O5 and O6, psychrotrophic were lower than mesophilic counts for all other samples. The proportion of psychrotrophs (ratio of psychrotrophs/total bacterial counts) increased notably between the two sampling days /day 0 and day 4) for most samples: whereas, the initial proportion of psychrotrophs in samples C1, C2, C3, C5, C6, O1 and O2 was below 20%, considerable higher proportions were determined from C4 (50%), O3 (60%), O4 (80%), O5 (40%) and O6 (100%) at day 0. After 4 days storage at 4°C, psychrotrophic bacteria largely dominated in samples C1, C3, C4, C6, O1, 04, and 06 (100%), whereas the proportions ranged between 40 and 70% for C2, C5, O2, O3 and O5.

3.2 AR load evaluated by RAPD mean values

When combining all results from the different investigated ABs, following observations were made considering RAPD: for CP milk samples, the RAP0 mean values were similar for mesophilic (M) and psychrotrophic (P) populations, whereas for OP samples the RAP0 was slightly lower for mesophiles, contrarily to psychrotrophs for which it was highest (0.245) (Fig.2).

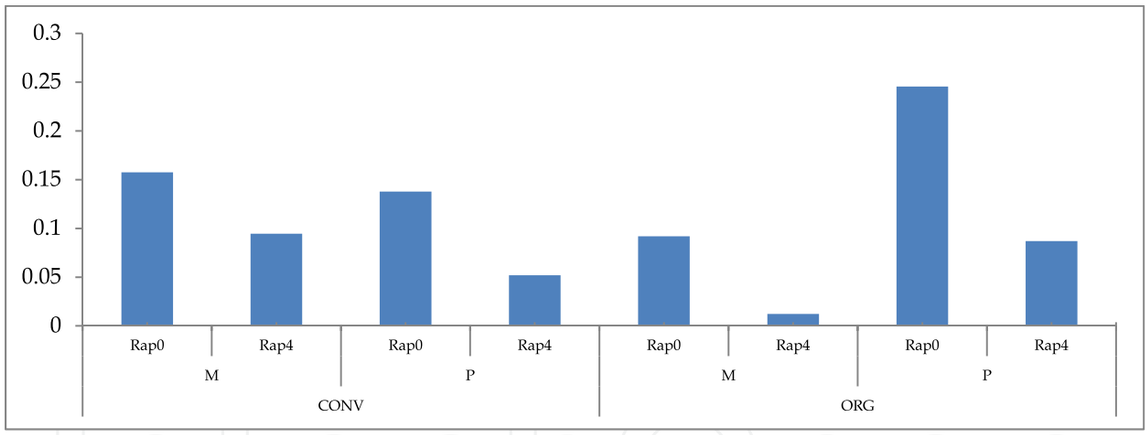


Fig. 2. Mean RAPD values from CP and OP raw milk samples at sampling days 0 or 4, determined for mesophiles (M) and psychrotrophs (P).

All RAP4 mean values were lower than the corresponding RAP0 values, irrespective of the origin of the samples or the bacterial population types. The major drop was observed for psychrotrophic populations retrieved from OP milk samples. For the mesophiles, the AR evaluated through RAPD clearly indicated a decrease for both types of samples, even though more important for bacteria retrieved from OP samples.

3.3 Comparison of RAPD mean values to evaluate the impact of cold storage

The trend of RAPDs were obtained by the results of the NPAR1WAY/REGW procedure (introduced in section 2.3.5). A typical example of the SAS-output where RAP4 was compared to RAP0 is detailed in Table 1.

Wilcoxon Scores (Rank Sums) for Variable rep
Classified by Variable day

Day	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	12	127.0	150.0	15.060747	10.583333
4	12	173.0	150.0	15.060747	14.416667

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	127.0000
Normal Approximation	
Z	-1.4939
One-Sided Pr < Z	**0.0676**
Two-Sided Pr > |Z|	0.1352
t Approximation	
One-Sided Pr < Z	**0.0744**
Two-Sided Pr > |Z|	0.1488

Z includes a continuity correction of 0.5.

Table 1. Output obtained with the Wilcoxon test applied to mesophiles present in CP raw milk samples, for one AB (G) at concentration I. In this example, RAP0 = 10.58 and RAP4= 14.42, however the t approximation value of 0.1488 indicated that RAP0 and RAP4 were statistically equivalent.

The conclusions from each comparison are summarised in Table 2 a,b. The mean RAP4 values only exceeded the mean RAP0 values for TS (red colour), at both concentrations, for psychrotrophic (P) populations retrieved from CP samples (Table 2a); with the exception of mesophiles (M) enumerated on C-containing plates, for which the RAP4 were lower than the RAP0 values (green colour), in all other conditions (yellow colour), the relative AR levels were equivalent. On the side of the populations retrieved from OP raw milk samples, for half of the conditions, RAP4 values were lower (green colour) or equal (yellow colour) to RAP0; but RAP4 widely exceeded RAP0 for 8 conditions (red colour), mostly for psychrotrophs (Table 2b).

3.4 Mean RAP4 from OP compared to mean RAP4 from CP raw milk samples

The comparison of the mean RAP4 values indicated that for 10 cases out of 16, the AR levels were similar after 4 days storage (yellow colour), irrespective of the milk type; mesophilic populations retrieved on C-containing plates, as well as mesophiles and psychrotrophs enumerated on G-plates (lower concentration, II) from OP samples carried less AR features (green colour)(Table 3); but, psychrotrophs from OP samples, enumerated on L (II) and TS-plates (I), exhibited much superior levels of AR as compared to CP raw milk samples (red colour).

3.5 Ranking of the four considered ABs

For both CP or OP samples, the ranking of the ABs was obtained with REGW based analyses that followed ANOVA. An example is given in Table 4 (a,b).

a)

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	>

b)

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	>>

Table 2. Mean RAP4 values compared to the mean RAP0 values from CP (a) and OP (b) raw milk samples, for the four ABs tested at concentrations I and II (I>II) for mesophiles (M) or psychrotrophs (P). The symbols =, <, and > are meaning RAP4 equalled, or was significantly below or superior to RAP0, respectively.

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	=

Table 3. Mean RAP4 values from OP compared to mean RAP4 values from CP raw milk samples, determined for each AB at concentrations I and II (I>II), for mesophiles (M) or psychrotrophs (P). The symbols =, <<, and >> are meaning that RAP4 from OP samples were significantly and respectively equal, much inferior or superior to RAP4 determined for the CP raw milk samples.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	32	61772.45833	1930.38932	**10.78**	**<.0001**
Error	63	11279.54167	179.04034		
Corrected Total	95	73052.00000			

R-Square	Coeff Var	Root MSE	RAP4 Mean
0.845596	27.58886	13.38060	48.50000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AB	3	30643.27083	10214.42361	57.05	<.0001
dose	1	7315.04167	7315.04167	40.86	<.0001
ech	5	8529.00000	1705.80000	9.53	<.0001
AB*dose	3	3033.89583	1011.29861	5.65	0.0017
AB*ech	15	9065.47917	604.36528	3.38	0.0004
dose*ech	5	3185.77083	637.15417	3.56	0.0067

a)

Alpha	0.05		
Error Degrees of Freedom	63		
Error Mean Square	179.0403		
Number of Means	2	3	4
Critical Range	8.8494249	9.2716373	10.19334

Means with the same letter are not significantly different.

	Mean	N	AB
A	**65.125**	**24**	**4**
A	**62.354**	**24**	**2**
B	**46.375**	**24**	**1**
C	**20.146**	**24**	**3**

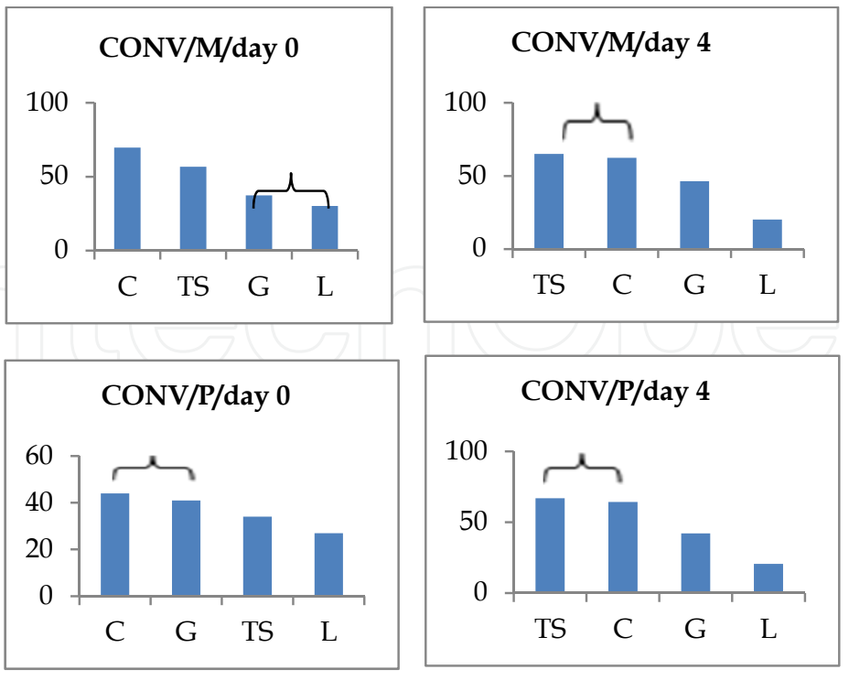
b)

a) Dependent Variable: RAP4 ; Values of RAP4 were replaced by ranks
b) Ryan-Einot-Gabriel-Welsch Multiple Range Test for RAP4
NOTE: This test controls the Type I experiment wise error rate.

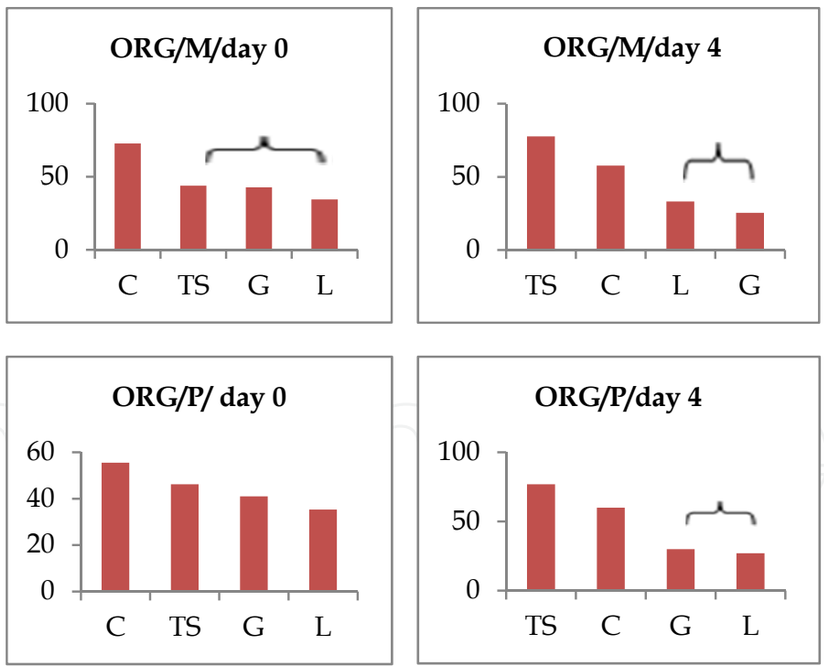
Table 4. a) Analysis of variance of RAP4 ranks from mesophiles present in CP samples. The R-square value was equal to 0.85 and the F p-value was < 0.0001, which was indicative of a good ANOVA model. All three effects (AB, dose, sample /ech) and their interactions were influential with a predominant and significant effect of the AB. b) Output from the REGW test. For the same samples, AB4 (TS) and AB2(C) had equivalent effects, higher to AB1(G) and to AB3 (L); AB1 and AB3 were not equivalent.

No important differences distinguished CP and OP raw milk samples when considering the ranking of the four ABs according to their respective prevalence for mesophiles (M) or psychrotrophs (P) (Fig. 3a, b).

The same ranking, with a preponderant C-resistance was characteristic of day 0 and was observed for all populations types retrieved whether from CP or OP raw milk samples; a slightly higher AR for TS was observed for mesophiles from CP samples, whereas for G and L a similar ranking was noticeable for both sample types. Higher AR was recorded for psychrotrophs from OP samples compared to CP samples for C, TS and L, with the exception of G for which similar levels were observed (Fig. 3a, b). At day 4, the same



a)



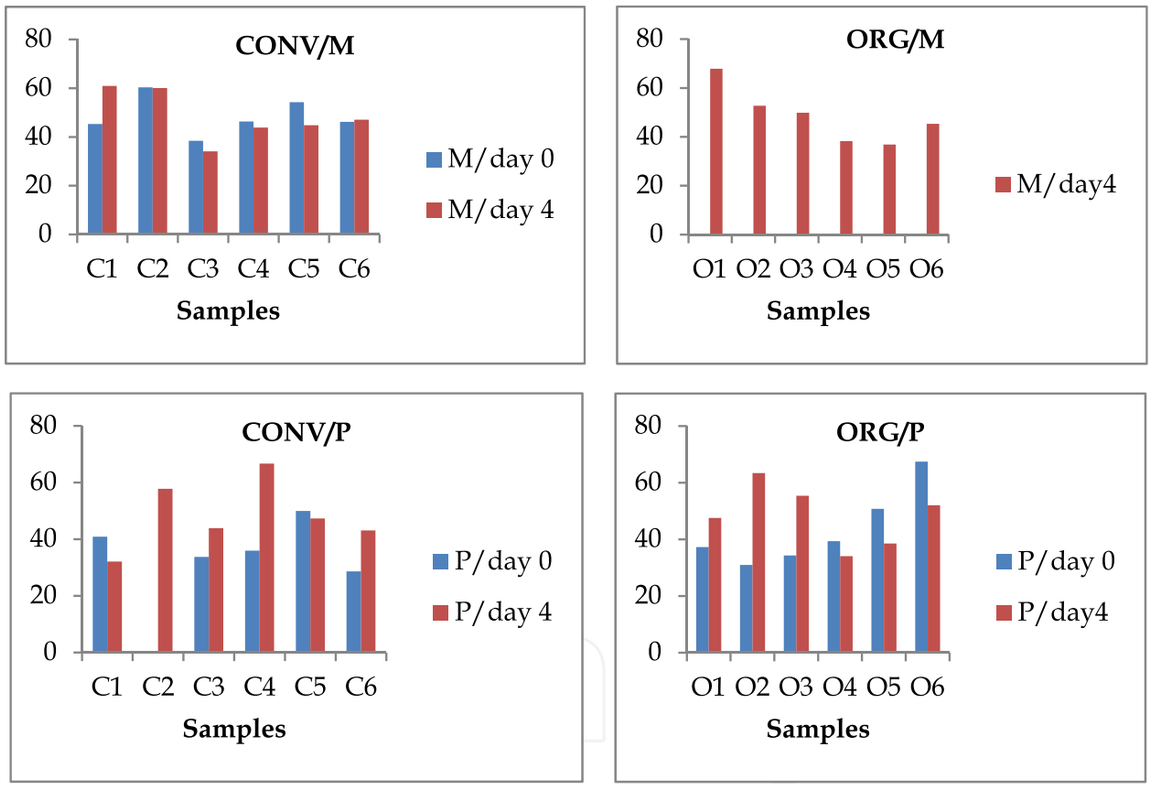
b)

Fig. 3. AR prevalence, estimated by multiple comparison of means from CP (a) and OP (b) raw milk samples over time, determined from combined results with both concentrations (I and II) of each AB. The mean values were obtained with the REGW multiple range test for RAP0 and RAP4. [Noteworthy, ABs that were similarly ranked are grouped in a bracket].

ranking of the ABs was observed for TS and C; the levels were equivalent for psychrotrophs and mesophiles from CP samples (Fig. 3a) whereas TS supplanted C for both mesophiles and psychrotrophs from OP samples (Fig. 3b). For G, similar levels of AR were recorded from CP samples for both psychrotrophs and mesophiles at days 0 and 4, in contrast to OP samples in which the AR dropped over time by about half for both types of bacterial populations (Fig. 3a,b). L-resistance was least prevalent at day 0, mostly also at day 4; a decrease of AR was recorded over time for mesophiles from CP samples and psychrotrophs from CP and OP raw milk samples.

3.6. Ranking of the milk samples according to their total AR load

At day 0, the AR levels (as means determined with the Ryan-Einot-Gabriel-Welsch multiple range test) were comprised between 38-60 and 28-50 for mesophiles and psychrotrophs from CP samples, respectively (Fig. 4a).



(a) (b)

Fig. 4. AR load from multiple comparison of sample means for CP (a) and OP (b) raw milk samples at days 0 and 4 determined for mesophiles (M) and psychrotrophs (P).

At day 4, the mean values ranged between 32-60 and 32-67 for the mesophiles and psychrotrophs respectively (Fig. 4a). Mesophiles from samples C3, C4, C5 showed moderate decrease of the AR between days 0 and 4, contrarily to the psychrotrophic populations recovered from the same samples, which increased by 10 and 30 for samples C3, C4 respectively, or slightly decreased for C5 (Fig. 4a). Mesophiles from sample C2 were the

highest AR carriers at days 0 and 4 whereas sample C3 was the lowest at both sampling days; sample C1 was the second lowest at day 0, and second highest at day 4. No model enabled to rank the six OP samples at day 0; however at day 4, the comparison between mesophiles and psychrotrophs showed that samples may be similarly ranked over time (like O3, O5) or not (sample O1), (Fig. 4b).

3.7 DGGE profile analyses of CP and OP raw milk samples at sampling days 0 and 4.

At day 0, the primer set yielded relatively complex fingerprints for C1, C2, C3, C4, C5, C6, O1, O2, and O3 as the PCR amplicons migrated along the whole length of the denaturing gradient gel (Fig. 5), indicative of a high variability in GC%, hence a high species diversity, irrespective of the milk type.

Based on Gel Compar ™ analyses, some samples displayed profiles with as many as 27 or 26 bands for C6 and O1 respectively, whereas C4, O4, O5 displayed simpler profiles comprising only 10-11 bands. Profiles from CP samples (C1, C5, C6) that yielded the highest amount of bands clustered as well as O1, O2 and O3 from OP raw milk samples (Fig. 5), and showed similar community structure. The 4 days-cold storage implicated for most samples a simpler profile, as less bands were detected; with exception of C1, all CP raw milk samples clustered while the profiles of C5 and C6 still formed a sub-cluster. Cold storage affected O1, O2 and O3 differently: the samples O1 and O2 remained clustered despite a drop of the similarity values (77.2% to 52.3 %) between both sampling days (Fig. 5).

Samples O4, O5 and O6 were least affected by cold storage, as all three samples formed almost exact pairs at days 0 and 4 with similarity values of 75%, 88.5%, and 87.5% for O6, O5 and O4 respectively, indicative of small changes in the bacterial community between the sampling days. For most of the samples, following the cold storage (4°C for 4 days), the banding patterns were more located on the top of the gel, indicative of less bacterial diversity among communities, and domination of species with higher AT%. To some extent, CP and OP samples were distinguished by DGGE analyses.

4. Discussion

The statistical analyses of RAPD means and RAPD trends during cold storage revealed 1) a higher AR level for psychrotrophic populations present in OP compared to CP milk samples at day 0 (Fig. 2); 2) a similar drop of C-resistant mesophilic, and raise of TS-resistant psychrotrophic bacteria over time for both types of samples (Table 2a,b); 3) an increase of L-resistant (at both concentrations) and partial increase (at one concentration) of G-resistant psychrotrophs for OP samples (Table 2a,b); 4) RAP4 from OP was lower to RAP4 from CP in 4 cases of 16, was similar to RAP4 from CP in 10 other tested conditions, but exceeded RAP4 from CP in 2 cases (Table 3). All preceding observations indicate that the AR load on bacterial populations from OP samples may be as high if not higher than for CP samples. When considering the farming type practices, small or no differences in AR levels were reported by Sato et al. (2004) for *Campylobacter* spp. isolates from organic and conventional dairy herds, by Roesch et al. (2006) when the AR of udder pathogens was investigated in dairy cows, by Ray et al. (2006) for *Salmonella*, or by Garmo et al. (2010) for coagulase-negative staphylococci: the frequency of AR in organic farms was not so different from

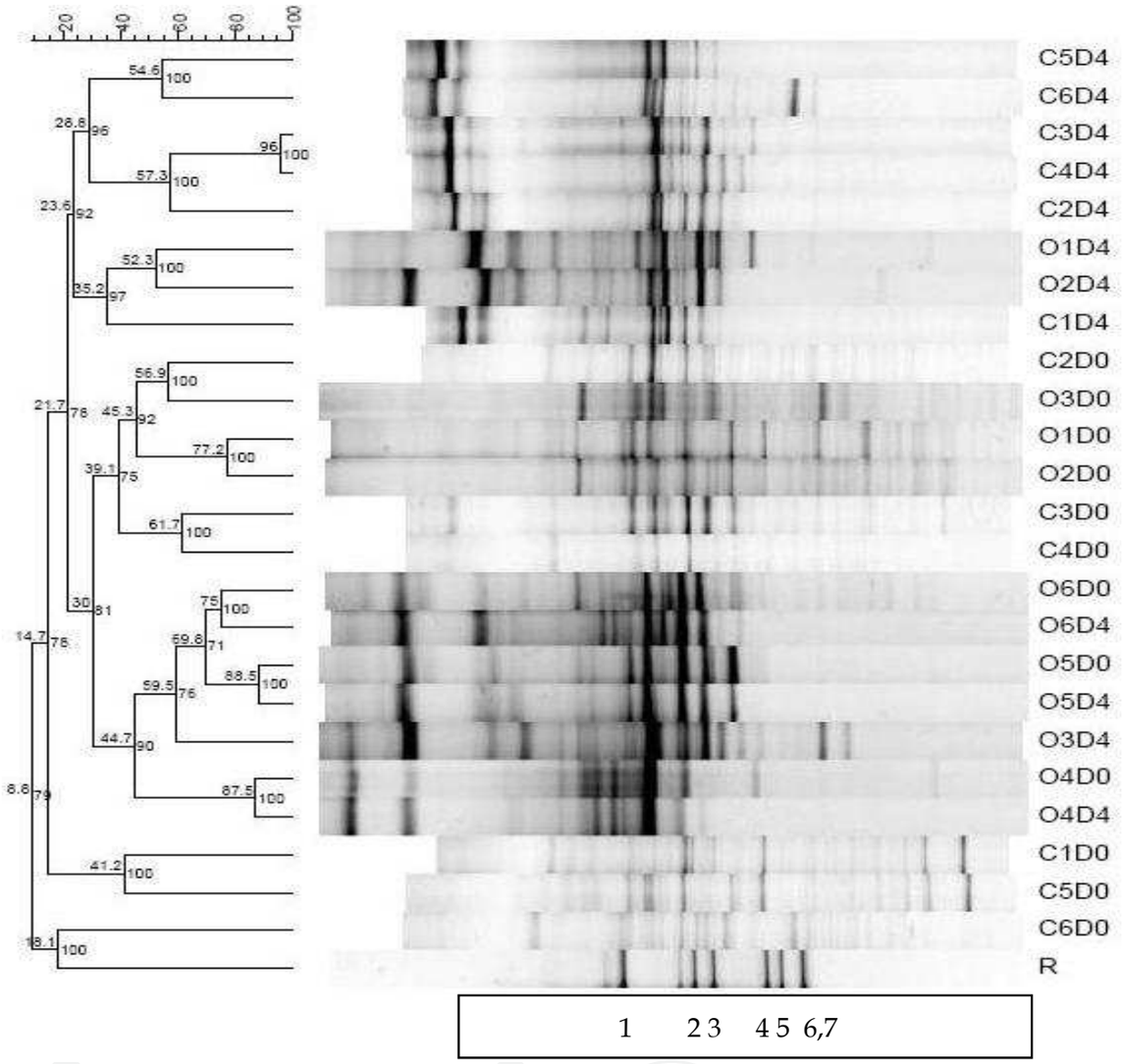


Fig. 5. Dendrogram of DGGE profiles from CP (C1 to C6) and OP (O1 to O6) raw milk samples determined at day 0 (D0) and after 4 days storage at 4°C (D4). The reference standards include the following species: 1, *Listeria innocua* CCUG 15531^T (ATCC 33090) ; 2, *Acinetobacter johnsonii* HAMBI 1969 (ATCC 17909) ; 3, *Pseudomonas tolaasii* LMG 2342^T (ATCC 33618); 4, *Bacillus cereus* HAMBI 250 (ATCC 10987) ; 5, *Escherichia coli* HAMBI 99 (ATCC 11775) ; 6, *Stenotrophomonas maltophilia* HAMBI 2659 (ATCC 13637) ; 7, *Burkholderia cepacia* HAMBI 1976 (ATCC 25416). [ATTC, American Type Culture Collection; CCUG, Culture Collection University of Göteborg; HAMBI, Culture Collection of the University of Helsinki; LMG, Belgian Collection of Microorganisms].

conventional farms. Also Ruimy et al. (2010) recorded similar levels of resistant Gram-negative bacteria for both organic and conventional produced fruits and vegetables. However, other studies report still higher susceptibilies to antibiotics for samples originating from organic production systems.

Compared to RAP0 values obtained for 18 CP samples (analysed in a previous study), which ranged between 0.0788 and 0.1576, it appeared that the mesophylic populations from organic raw milk samples analysed here had a rather low AR load (0.092), contrarily to the psychrotrophs for which the mean RAP0 value 0.2456 was the highest so far observed for all milk samples investigated and for which the mean values ranged between (0.1378-0.158). The RAP4 value from OP samples (0.0869) was still the highest encountered for psychrotrophs among all so far investigated raw milk samples for which the RAP4 values were comprised between 0.030 and 0.051 (Fig. 2, and Munsch-Alatossava et al. 2012).

In this study, irrespective of the milk type or the investigated bacterial population types (psychrotrophs or mesophiles), C-resistance was most prevalent at day 0 (rather fresh milk) whereas TS-resistance was equivalent to C-resistance (for CP samples) or higher (for OP samples) at day 4 (after the milk underwent a longer cold storage) (Fig. 3a, b). Similar observations were made in the previous study that considered 18 raw milk samples from conventional production systems (Munsch-Alatossava et al. 2012) for which TS-resistance supplanted C-resistance in prevalence following the cold storage. To some extent, AR prevalence at day 0 does mirror AB usage: Kools et al. (2008) in an overview of published data around ABs usage in Europe, indicated the following relative proportions of total AB use for Finland: β-lactams and cephalosporins (62%), Sulfonamides and trimethoprim (16%), Aminoglycosides (2%) and fluoroquinolones/quinolones (0.6%).

By summing up the values obtained from the ranking of the four ABs according to their prevalence at each sampling day and for each population type (Fig. 3a,b), the total of the means (from the REGW test) from each AB reached 194 for mesophiles from both CP and OP raw milk samples at days 0 and 4. Also, psychrotrophs from OP samples exhibited the same level of total AR at day 4 (194), against 178 at day 0. For psychrotrophs from CP samples, the total of the means from each AB was 146 and 184 at days 0 and 4, respectively. From the analysed samples, it appeared that the multiresistant trait was as common among mesophilic bacteria whether they originated from CP or OP samples. Compared to mesophiles, multiresistance seemed to be less common among psychrotrophs at day 0; however, the cold storage permitted a significant raise of the number of multiresistant isolates (more important for CP samples), but the total AR reached a superior value for psychrotrophs from OP samples at day 4. The AB ranking revealed a notable different impact of the cold storage on the AR trend considering the investigated bacterial population types (Fig. 3a, b):

- since the total AR loads were around 194 for both milk types over time, changes at the level of mesophiles appeared to be mainly qualitative (for example, replacement of bacterial species rather resistant to C by species rather resistant to TS)
- changes at the level of psychrotrophs were both qualitative (TS resistance was more frequent than C resistance at day 4 for OP samples), but also quantitative as suggested from the increase of the AR load by respectively 38 and 16 units for psychrotrophs from CP and OP systems during cold storage; the increase may be lower in the later case due to the particular features of samples O4, O5 and O6.

Interestingly, the statistical analyses of RAPD, which enabled a sample ranking according to the "AR loads" revealed that samples may be or not similarly ranked over time following the cold storage, depending on the considered populations (whether mesophiles or psychrotrophs)(Fig. 4a,b). Noteworthy, samples O5 and O6 were ranked as highest AR

carriers on the side of the psychrotrophs at day 0 whereas, at day 4, a partition into two classes considered O3, O1 and O2 as high AR carriers, and O6, O5 and O4 as lower ones. Clearly, the cold storage affected differently OP samples while for samples O1, O2 and O3 the AR, estimated as relative amounts, increased, the AR decreased for O4, O5 and O6.

The high RAP0 and RAP4 values of the six considered OP samples (Fig. 2) bring up the question on whether the AR load is the consequence of different milk production practices or may be sample dependant. Part of the answer is given by the RAP0 and RAP4 values, together with their respective variation ranges: the RAP0 and RAP4 variation ranges were comprised between 0-12 (mean 0.2456) and 0-0.996 (mean 0.0869) respectively, for psychrotrophs enumerated from OP samples. In a previous study where the AR was followed over time (at days 0, 2 and 4 of cold storage) from CP raw milk samples it was noticed that RAP2 (determined after 2 days cold storage) and the corresponding variation range largely supplanted RAP0 and RAP4 values and their corresponding variation ranges (Munsch-Alatossava et al. 2012). Moreover ANOVA performed on RAP0 and RAP4 ranks revealed significant main effect (F values from the Fischer-Snedecor test, $p<0.0001$ were highest) of the "AB type" for all analysed conditions (day0/day4; mesophiles/psychrotrophs; conventional/organic production systems) to the exception of psychrotrophs from organic milk samples for which the "sample" was the most important factor.

Consequently, we hypothesize that since total counts for O4 and O5 were highest at day 0 (around and slightly above 10^5 CFU/ml, Fig. 1), lower for O6 which however exhibited the highest percentage of psychrotrophs at day 0 (100%), the excessively high value of Rap0 may be due to the samples O4, O5 and O6, which were most probably longer cold stored prior to the initial analyses as compared to O1, O2 and O3. This point is reinforced by the DGGE based analyses of OP samples, as for the samples O4, O5 and O6 the profiles at day 4 were quite similar as the ones of day 0, indicative of little changes in the bacterial community over time (Fig. 5). Like for other studies, DGGE based data confirmed the potential of this approach to investigate changes in raw milk at population levels. Clearly, the cold storage (4 days at 4°C) promoted a reduction of dominant bacterial species: similar observations were made with DGGE based analyses for raw milk stored at 4°C for 24h by Lafarge et al. (2004), but also for meat (Li et al., 2006).

To some extent, results from DGGE analyses were coherent with plating results: the samples C1, C5, C6, O1 and O2 showed the highest bacterial diversity at day 0 as visible from the most complex banding patterns (Fig. 5). The same samples presented the lowest percentages of psychrotrophs in the initial microflora at day 0 (data not shown), suggesting that these samples were more fresh, and had the lowest storage history. Conversely C4 and O4, for which high percentages of psychrotrophs were recorded at day 0, yielded electrophoretic patterns with fewest bands amounts (Fig. 5).

What could explain such high if not higher AR levels in bacteria from organic compared to conventional production systems? Even though any use of antimicrobials may create the potential for AR development, at day 0, the usage patterns of ABs could somehow constitute one part of the explanation (Kools et al., 2008; Thomson et al., 2008). Manure contains substantial amounts of both antimicrobials and antimicrobial-resistant microorganisms; agricultural practices that prevail in organic production systems where chemical fertilizers are prohibited, and are replaced by antibiotic-polluted manure applications, and where at

least 50% of the feed is produced on the farm may also partially explain rather high AR levels in OP compared to CP samples. As milk is the target of numerous sources of contaminations (soil, environment…), it may be not that surprising if AR levels are at least as high for OP as for CP samples .

In 2008, the European Commission launched the European Union ´s new organic farming campaign under the slogan "Organic farming, Good for nature, Good for you". At the same time, the European Food Safety Authority (2008) attempted to evaluate to which extent food serves as a source for the acquisition by human of antimicrobial-resistant bacteria and whether foodborne antimicrobial resistance constitutes a biological hazard.

The statistics applied for data treatment on ranks of RAPD which compared the AR load in milk over time as quantified through RAPD (indicative of relative amounts) suggested that the AR level, the AR trend over time may be less "milk production type" than "sample" dependant; the main determinant may be the initial microflora. Whether cold storage of raw milk promotes the raise of AR, of multiresistant traits among bacteria and whether it affects differently conventionally or organic produced milk needs still to be further investigated. The image of safer and healthier food is most often associated to organic food by consumers. Whether initial good intentions based on a more rare use of ABs (typical for organic production systems), contrarily to conventional systems with more frequent use of ABs, are diverted by microbial activity needs further clarifications.

5. Conclusion

In this study, AR was highest at day 0 for psychrotrophs present in OP samples. Even though the cold storage globally promoted a drop of RAP values (as relative amount of AR) over time, in detail the trends were more contrasted as the AR load increased for psychrotrophs from OP samples for both L and TS at both tested concentrations, during the 4 days storage at 4°C. The AR only dropped for mesophiles, from both sample types, for C at both concentrations. For OP samples, if C-resistance was most frequent at day 0 (which corresponds to rather fresh milk), TS-resistance was more common at day 4 (when the milk already underwent a certain cold storage). Based on DGGE pattern analyses, the bacterial communities fingerprints appeared to be at both sampling days ¨milk age¨ and ¨milk type¨ dependant as the clustering distinguished CP and OP raw milk samples, with different freshness e.g. more or less cold stored. Moreover the changes in bacterial populations structure, following cold storage, indicated a shift in banding patterns towards AT-rich regions, suggesting that the cold storage of raw milk promotes the dominance of AT-rich species over time, irrespective of the milk type.

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7. References

Aminov, R.I. (2009). The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology,* Vol.11, pp. 2970-88.

Chambers, J.V. (2002). The microbiology of raw milk, In: *Dairy Microbiology Handbook, 3rd edition*. Edited by R. K. Robinson, New York: Wiley-Interscience, pp. 39-89.

Conover, W.J. (1980). *Practical Non Parametrics Statistics*, 2nd edition, John Wiley & Sons; New York.

Cousin, M.A. (1982). Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. *Journal of Food Protection,* Vol. 45, pp. 172-207.

Davies, J.& Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*, Vol. 74, pp. 417-433.

Einot, I. & Gabriel, K.R. (1975). A study of the power of several methods of multiple comparisons. *Journal of the American Statistical Association*, Vol. 70 (351), pp. 574-583.

European Commission (2008). Organic Farming-What makes milk organic? www.organic-.farming.europa.eu.

European Committee for antimicrobial susceptibility testing (EUCAST), (2000). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. *CMI* Vol.6, pp.509-515.

European Council Regulation (EC) No834/2007 of June 2007 on organic production and labelling of organic products and repealing regulation (EEC) No2092/91.

European Food Safety Authority (2008). Foodborne antimicrobial resistance as a biological hazard (Question No EFSA-Q-2007-089) by the Biohaz panel (Andreoletti et al.)

Finnish association for milk hygiene. (2008) http://www.maitohygienialiitto.fi

Finnish Food safety authority (2008) http://www.evira.fi

Freitas, R.; Nero, L.A. & Carvalho, A.F. (2009). Technical note: Enumeration of mesophilic aerobes in milk: Evaluation of standard official protocols and Petrifilm aerobic count plates. *Journal of Dairy Science*, Vol. 92, pp. 3069-3073.

Garmo, R.T.; Waage, S.; Sviland, S.; Henriksen, B.F.; Østerås, O. & Reksen, O. (2010). Reproductive performance, udder health, and antibiotic health, and antibiotic resistance in mastitis bacteria isolated from Norwegian red cows in conventional and organic farming. *Acta Veterinaria Scandinavica,* Vol. 52, pp. 1-13.

Gray, M.J.; Freitag, N.E. & Boor, K.J. (2006). How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect ion and Immunity,* Vol. 74, pp. 2505-2512.

Grave, K.; Torren-Edo, J. & Mackay, D. (2010). Comparison of the sales of veterinary antibacterial agents between 10 European countries. *Journal of Antimicrobial Chemotherapy*, Vol. 65, pp. 2037-2040.

Information Centre of the Ministry of Agriculture and Forestry (2009). Matilda tietopalvelu. Maito and kananmunat. http://www.matilda.fi.

Jacob, M.E.; Fox, J.T.; Reinstein, S.L. & Nagaraja, T.G. (2008). Antimicrobial susceptibility of foodborne pathogens in organic and natural production systems: an overview. *Foodborne Pathogens and Disease*, Vol. 5, pp. 721-730.

Knezevic, P. & Petrovic, O. (2008). Antibiotic resistance of commensal *Escherichia coli* of food-producing animals from three Vojvodinian farms, Serbia. *International Journal of Antimicrobial Agents*, Vol. 31, pp. 360-363.

Kools, S.A.E., Moltmann, J.F. & Knacker, T. (2008). Estimating the use of veterinary medicines in the European union. *Regulatory Toxicology and Pharmacology*, Vol. 50, pp. 59-65.

Lafarge, V. ; Ogier, J.-C.; Girard, V.; Maladen, V.; Leveau, J.-Y.; Gruss, A. & Delacroix-Buchet, A. (2004). Raw cow milk bacterial population shifts attributable to refrigeration. *Applied and Environmental Microbiology,* Vol. 70, pp. 5644- 5650.

Levy, S.B. (2002). Factors impacting on the problem of antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, Vol. 49, pp. 25-30.

Li, M.Y; Zhou, G.H.; Xu, X.L. ; Li, C.B. & Zhu,W.Y.(2006). Changes of bacterial diversity and main flora in chilled pork during storage using PCR-DGGE. *Food Microbiology*, Vol.23, pp. 607-611.

Ma, Y.; Barbano, D.M. & Santos, M. (2003). Effect of CO_2 addition to raw milk on proteolysis and lipolysis at 4 °C. *Journal of Dairy Science,* Vol. 86, pp. 1616-1631.

Marshall, B.M.; Ochiend, D.J. & Levy, S.B. (2009). Commensals: Underappreciated reservoir of antibiotic resistance. *Microbe,* Vol. 4, pp. 231-238.

Munsch-Alatossava, P. & Alatossava, T. (2006). Phenotypic characterization of raw milk-associated psychrotrophic bacteria. *Microbiological Research*, Vol. 161, pp. 334-346.

Munsch-Alatossava, P. & Alatossava, T.(2007). Antibiotic resistance of raw-milk associated psychrotrophic bacteria. *Microbiological Research,* Vol. 162, pp. 115-123.

Munsch-Alatossava, P.; Gursoy, O. & Alatossava, T. (2010). Potential of nitrogen gas (N_2) to control psychrotrophs and mesophiles in raw milk. *Microbiological Research*, Vol. 165, pp. 122-132.

Munsch-Alatossava, P.; Gauchi, J.-P.; Chamlagain, B. & Alatossava, T. (2012). Trends of antibiotic resistance in mesophilic and psychrotrophic bacterial populations during cold storage of raw milk, ISRN Microbiology, In Press.

Ogier, J.C.; Son, O. ; Gruss, A.; Tailliez, P. & Delacroix-Buchet, A. (2002). Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Applied and Environmental Microbiology*, Vol. 68, pp. 3691-3701.

Ray, K.A.; Warnick, L.D.; Mitchell, R.M.; Kaneene, J.B.; Ruegg, P.L.; Wells, S.J.; Fossier, C.P.; Halbert, L.W. & May, K. (2006). Antimicrobial susceptibility of *Salmonella* from organic and conventional dairy farms. Journal of Dairy Science, Vol. 89, pp. 2038-2050.

Roesch, M.; Perreten, V.; Doherr, M.G.; Schaeren, W.; Schällibaum, S. & Blum, J.W. (2006). Comparison of antibiotic resistance of udder pathogens in dairy cows kept on organic and on conventional farms. *Journal of Dairy Science*, Vol. 89, pp. 989-997.

Ruimy, R.; Brisabois, A.; Bernede, C.; Skurnik, D.; Barnat, S.; Arlet, G.; Momcilovic, S.; Elbaz, S.; Moury, F.; Vibet, M.-A.; Courvalin, P.; Guillemot, D. & Andremont, A. (2010). Organic and conventional fruits and vegetables contain equivalent counts of Gram-negative bacteria expressing resistance to antibacterial agents. *Environmental Microbiology*, Vol. 12, No.3, pp. 608-615.

Ryan, T.A. (1959). Multiple comparisons in psychological research. *Psychological Bulletin*, Vol. 56, pp. 26-47.

Ryan, T.A. (1960). Significant tests for multiple comparisons of proportions, variances and other statistics. *Psychological Bulletin*, Vol. 57, pp. 318-328.

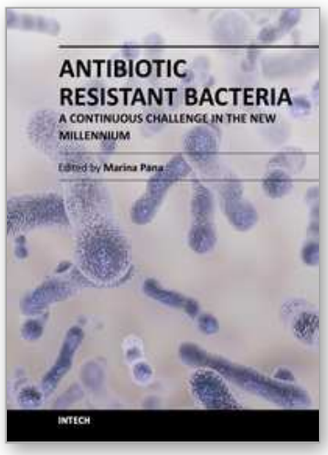
Sato, K.; Bartlett, P.C.; Kaneene, J.B. & Downes, F.P. (2004). Comparison of prevalence and antimicrobial susceptibilities of *Campylobacter* spp. isolates from organic and conventional dairy herds in Wisconsin. *Applied and Environmental Microbiology*, Vol. 70, pp. 1442-1447.

Schoeni, J.L.& Lee Wong, A.C. (2005). *Bacillus cereus* food poisoning and its toxins (Review). *Journal of Food Protection,* Vol. 68, pp. 636-648.

Straley, B.A.; Donaldson, S.C.; Hedge, N.V.; Sawant, A.A.; Srinivasan, V.; Oliver, S.P. & Jayarao, B.M.(2006). Public health significance of antimicrobial- resistant gram-negative bacteria in raw bulk tank milk. *Foodborne Pathogens and Disease*, Vol. 3, pp. 222-233.

Thompson, K.; Rantala, M.; Hautala, M.; Pyörälä, S. & Kaartinen, L. (2008). Cross-sectional prospective survey to study indication-based usage of antimicrobials in animals: Results of use in cattle. *BMC Veterinary Research*, Vol. 4, pp. 15-21.

Welsch, R.E. (1977). Stepwise multiple comparison procedures. *Journal of the American Statistical Association*, Vol. 72 (359), pp. 566-575.



Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium

Edited by Dr. Marina Pana

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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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