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Final report on the effect of microbial consortia on the sensorial qualities of cheeses and development of *Listeria monocytogenes*

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Report on the effect of microbial consortia on the sensorial qualities of cheeses and development of *Listeria monocytogenes*

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Summary

In this demonstration task, the effect of a simplified anti-*Listeria* consortium, composed of lactic acid bacteria and surface flora (based on a consortium exhibiting anti-*Listeria* activity in the core of cheese or at the surface during experimental ripening conditions) was evaluated in real cheese productions. In a first time, the incidence of the selected consortium on sensorial properties and *L. monocytogenes* growth was evaluated in 2 different cheese technologies: raw milk Saint-Nectaire and pasteurized milk Pont l'Evêque.

For Saint-Nectaire, the addition of the anti-*Listeria* consortium had no marked anti-*Listeria* effect in the raw milk cheeses manufactured in the farm selected for this study. The growth of *Listeria monocytogenes* and lag phase were different from a day of manufacturing to another. The inoculation of microbial consortium had an effect on sensorial properties of Saint-Nectaire cheeses. Saint-Nectaire cheeses with consortium addition had the highest scores for general intensity and pungent, acid and mouldy aroma, less hard cooked cheese and dried fruit aromas and were more sticky and melting than those without consortium.

Concerning Pont-l'Evêque cheeses, the microbiological and physico-chemical analyses as well as sensory evaluation did not showed significant differences between the "control" and "assay" samples. For sensorial properties, the most significant differences were rather correlated to the production date. At 38 days the *L. monocytogenes* count was 1.5Log lower in assay cheese than in control cheeses. But the Best Before Date, no difference for *L. monocytogenes* was observed between the « controls » and the inoculated cheeses ("assays").

The low incidence of the consortium on *L. monocytogenes* observed for Saint Nectaire and Pont-l'Evêque cheeses might have been related to the fact that the inoculation was performed at the surface of the cheese (simulating a post-contamination) whereas this consortium was developed for the inhibition in the core of cheese. However, the rather good implantation of the consortium strain (especially for lactobacilli) suggested that it might be more suited for milk contamination by *Listeria* (raw milk). This was confirmed, in a second time, by experiment on a third type of cheese technology. In Cantal cheeses prepared in a farm presenting recurrent cheese contamination by *L. monocytogenes*, this bacteria was no more detected after 3 months in cheeses with consortium while the pathogen was still present in some samples without consortium.

These last results open perspectives for the use of the selected anti-*Listeria* consortium as a preventive tool in raw milk cheese-producing farms confronted with recurrent *L. monocytogenes* contamination of their milk.

Introduction and objectives

The guarantee of cheese safety (inhibition of *Listeria monocytogenes*) without affecting their sensorial properties (flavour, aspect) is a major goal for traditional cheese producers. In this purpose, in task 2 of WP2A of the Truefood project, microbial consortia or strains isolated from milks or cheeses and presenting with antagonistic activities toward *L. monocytogenes* have been studied. A consortium was defined by simplifying a complex consortium issued from raw milk. It exhibited similar high anti-*Listeria* activities as the shown by the complex consortium. It is composed of lactic acid bacteria (6 species of *Lactobacillus* and *Leuconostocs*), *Staphylococcus* (2 species) and *Corynebacteria* (5 species) able to inhibit the development of *Listeria* in the core of cheese or at the surface in our experimental ripening conditions (cf deliverable D22.10 about “*Final report on efficiency of strains or consortia for inhibiting pathogenic bacteria*”). The optimisation and simplification of this consortium in order to use it in farm and small plant are described in deliverable D6.1.1.1 : “*Report describing the preparation of microbial consortium adapted to farm and SME production*”. The efficiency of this consortium was tested only in a non-cooked model cheese. So, the objectives of this demonstration, before proposing a consortium to small cheese producers, were i) to evaluate its ease of use and efficiency in real conditions (plant or scale production), and ii) to test its effect on the sensorial properties of cheeses.

Therefore, the objectives of this task were :

- i to evaluate the consortium ease of use in a cheese production context
- ii to determine the efficiency of its anti-*Listeria* properties in real conditions (plant or scale production),
- ii to test their effect on the sensorial properties of cheeses.

To achieve these objectives from May 2009 to May 2010, the consortium was tested i) in a farm producing PDO St-Nectaire cheese prepared with raw milk, ii) in a farm producing Cantal and Salers PDO cheeses and iii) in a pilot plant producing pasteurized Pont-l’Evêque cheese. For Saint-Nectaire and Pont-l’Evêque, the surface cheeses were artificially contaminated with *L.monocytogenes*. For the consortium test in Cantal, the cheese was naturally contaminated by *L. monocytogenes*. The SME was largely involved in this demonstration from Saint-nectaire and Cantal cheeses as they have produced cheeses and ripened the cheeses without *L. monocytogenes*.

A Effect of microbial consortia on the characteristics and the sensorial qualities of St-Nectaire cheeses and the development of *Listeria monocytogenes*.

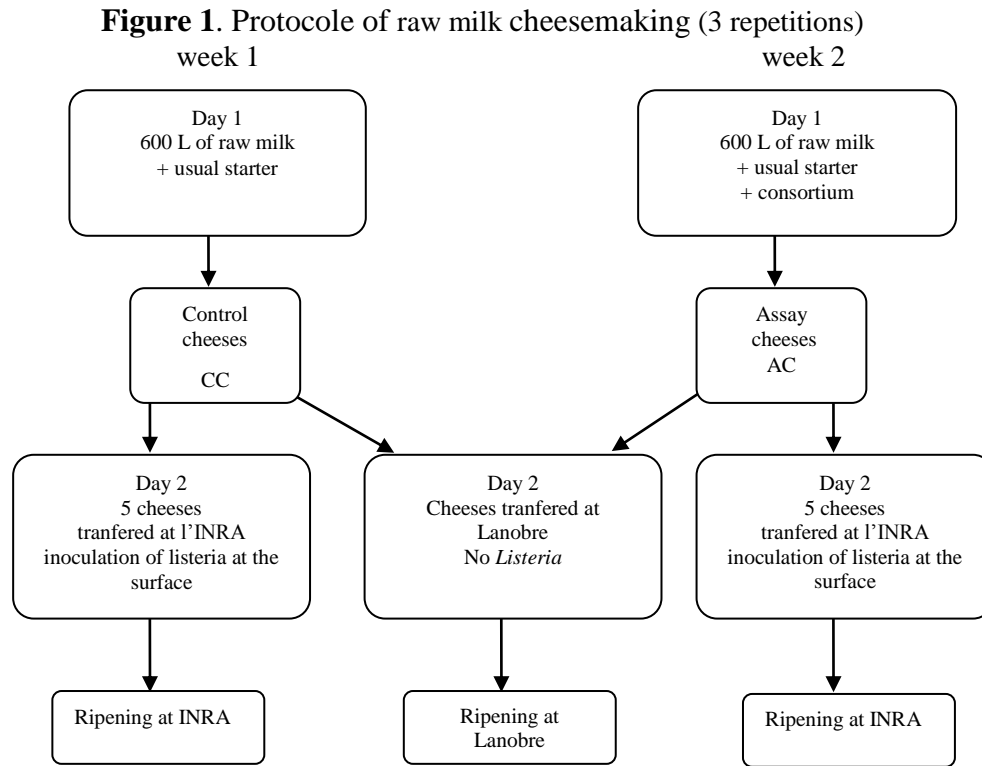
1. Methodology

1.1 Cheese making and strains inoculation

Three series of Saint-Nectaire cheeses were manufactured as described in figure 1. Cheeses were prepared with raw milk inoculated, just after milking, either with only the starter culture daily used by the farmer (control cheeses) or with cultures of the simplified anti-*Listeria* consortium (named AB) added to the starter (assay cheeses). The composition of this consortium and the level of inoculation of the different microbial flora were previously described (deliverable D6.1.1.1). It was composed of 17 strains belonging to two microbial

groups as described in Table 1: A (lactic acid bacteria), B (Gram positive and catalase positive bacteria).

The control and the assay were prepared the same week at different manufacturing days.



➤ **Anti-Listeria consortium composition and preparation**

Table 1. Composition of the simplified microbial consortium AB selected from raw milk: simplification of the microbial consortium (c deliverable D 6.1.1.1)

Strains	Level of inoculation in milk	
<i>Lactobacillus farciminis</i>	10 ⁴ cfu/ml	Group A Lactic acid bacteria
<i>Leuconostoc citreum</i>	10 ³ cfu/ml	
<i>Lactobacillus casei</i>		
<i>Lactobacillus curvatus</i>		
<i>Lactobacillus paracasei</i>		
<i>Lactobacillus plantarum</i>		
<i>Leuconostoc pseudomesenteroides</i>		
<i>Staphylococcus equorum</i>		
<i>Corynebacterium flavescens</i>		
<i>Arthrobacter nicotianae</i>		
<i>Corynebacterium casei</i>	10 ² cfu/ml	
<i>Brevibacterium linens</i>		
<i>Brachybacterium rhamnosum</i>		
<i>Macroccoccus caseolyticus</i>		

The strains were precultivated and inoculated as previously described in the deliverable D6.1.1.1. The levels of inoculation are indicated in table 1. They were inoculated with the starter cultures daily used by the farmer at the beginning of milking.

➤ *L. monocytogenes* inoculation

On day 2, 5 control cheeses (CC) and 5 assay cheeses (AC) of each manufacturing were inoculated at the surface with the strain S1 of *L. monocytogenes* previously used in WP2A, as described in the deliverable D6.1.1.1. The level of inoculation was 2 to 5 cfu/25 cm².

1.2 Cheese ripening conditions

Cheeses without *Listeria* were ripened in the same conditions than for the assays of task 2 of WP6 (28 days at 9°C and 98% hygrometry) in an industrial cellar (Lanobre). This cellar was the same than the one used for assays in task 6.1.2 of WP6.

Cheeses inoculated with *Listeria* were ripened in an INRA cellar in the same conditions described above. Control and assay cheeses were ripened in the same cellar. Temperature and humidity were automatically controlled by Air quality Process software and CRIC software.

1.3 Microbial analysis

L. monocytogenes was counted at the surface at 1, 4, 8, 13, 18, 23 and 28 days of ripened cheeses according to EN ISO/11290-2 by the accredited laboratory (Laboratoire Interprofessionnel du Massif Central LIAL, Aurillac, France).

Microbial populations were counted on different media : lactobacilli on the facultatively heterofermentative (FH) agar medium (Isolini et al, 1990), leuconostocs producing dextrans on Mayeux Sandine and Elliker (MSE) agar medium (Mayeux et al, 1962), enterococci on Slanetz and Bartley (SB) (Slanetz and Bartley, 1957), Gram positive catalase positive bacteria on Cheese Ripening Bacteria Medium (CRBM) (Denis et al, 2001), Gram negative bacteria on Plate Count Agar (FIL-IDF 100B, 1991) with crystal violet (1%) and vancomycin (0.5%) as inhibitor of the Gram positive bacteria (PCAI), and yeasts on Oxytetracyclin Glucose Agar (OGA) medium (Mossel et al, 1962).

1.4 Biochemical and physico-chemical analysis

The pH, the dry matter content (DM) and water activity (a_w) were measured in the core and at the surface of cheese after 1, 8, 18, 28 days of ripening. Dry matter content was determined according to the reference method NFV 046282 (12/95).

Lactose, glucose, galactose and organic acids, mainly lactic acid were measured by HPLC in the core and the surface of cheese after 1, 8, 18, 28 days of ripening as described by Picque et al, 2009.

1.5 Monitoring microbial dynamics

The dynamic of the different species was followed in the control and in the assay cheeses from manufacturing 1 by species or genus specific PCR (developed in task 2A and primers described in table 2) and by V2 and V3 rDNA SSCP analysis (Callon et al, 2007) on total DNA extracted from counting plates (FH, CRBM, MSE) or on total DNA extracted from cheeses at 8 and 18d of ripening.

Table 2 : Primers used for specific PCR

Genre ou espèce ciblée	Amorces	Séquences	Références
<i>Ln. mesenteroides</i>	Lnm1	5' TGTCGCATGACACAAAAGTTA 3'	Cibick et al, 2000
	Lnm2	5' ATCATTTCCCTATTCTAGCTG 3'	
<i>Ln. citreum</i>	Lncit1	5' ACTTAGTATCGCATGATATC 3'	Cibick et al, 2000
	Lncit2	5' AGTCGAGTTGCAGACTGCAG 3'	
<i>Corynebacterium casei</i>	Fs15	5' CCG CAA GGC TAA AAC TCA AAG GAA T 3'	Monnet et al., 2006
	Fs17	5' ACC GAC CAC AAG GGA AAG ACT 3'	
<i>Brevibacterium spp.</i>	brevifor	5' CGG TAC CTS CAG AAG AAG T 3'	Gelsomino et al., 2004
	brevirev	5' GTC AGT HAC AGC CCA GAG T 3'	
<i>Brachybacterium spp.</i>	Brachyfor	5' TCG GGA TAA CCT CGG GAA ATC 3'	Bournet 2008
	Brachyre	5' CGC ACG CCC GAG GTT G 3'	
<i>Macrocooccus caseolyticus</i>	Macfor	5'-TAG CTT CGC ATG AAG CAA TA-3'	Bournet 2008
	Macrev	5'-TTA CGA TCC GAA AAC CTT CTT-3'	
<i>Corynebacterium flavescens</i>	CF1	5'-GCC TTT TTT AAG GTG ACG GTA CCT-3'	Bournet 2008
	CF2	5'-ACA AGC CAT CTC TGA CCC AAT C-3'	
<i>Arthrobacter nicotianae</i>	ArtFor	5'-ATGACCTCGCACCCGCATGG-3'	Bournet 2008
	ArtRev	5'-TTTCGCTTCTTCCCTACTGAAAGAC-3'	
<i>Lb. plantarum</i>	16	5'-GCTGGATCACCTCCTTTC-3'	Berthier et al, 1999
	Lpl	5'-ATGAGGTATTCAACTTATG-3'	
<i>Lb. casei</i>	16Rev	5'-GAAAGGAGGTGATCCAGC-3'	Berthier et al, 2001
	paracasei 16S	5'-CACCGAGATTCAACATGG-3'	
<i>Lb. curvatus</i>	16	5'-GCTGGATCACCTCCTTTC-3'	Berthier et al, 1999
	Lc	5'-TTGGTACTATTTAATTCTTAG-3'	
<i>S. equorum</i>	SdAEqF	5'-GTGGAGGACACTTAAACCATTTC-3'	Corbière et al, 2004
	SdAEqR	5'-CCATTTACCATCGTTTACAACACTAG-3'	

1.6 Sensorial analysis

At the end of ripening (28 days), the sensorial qualities of cheeses (aroma, taste, texture and visual aspect of the surface) inoculated or not with the consortium and ripened at Lanobre (no inoculation with *Listeria*) were determined by a sensorial trained panel (12 judges) at the ENIL sensory laboratory. Thirty six descriptors of texture, aroma and taste were used to described cheeses, they were each noted in a scale 0 to 10.

1.7 Data treatments

Statistical analysis ANOVA (principal effect) were carried out at each time of the ripening using Statistica TM (version 5).

To determine the effect of consortium on *Listeria* counts, pH, DM, a_w , microbial flora, sugar and acid contents in the core and on the surface of cheeses ripened at INRA, two factors were taken into account : the inoculation (FE) or not (FC) of the consortium and the day of manufacturing (manufacturing 1, 2 and 3). A Principal Component Analysis (PCA) was performed at day 28 (surface) with only the significant data of the ANOVA. The same statistical analysis was applied on sensorial characteristics of 28 days old cheeses ripened at Lanobre to evaluate the effect of consortium and ripening conditions and a Principal Component Analysis (PCA) was realized at day 28 with the ANOVA significant data.

The results of species and genus specific PCR analysis were expressed as presence or absence of species in cheeses.

The SSCP profiles were analyzed by Genescan software and the ratio of each peak in profiles were calculated.

2. Results

2.1 Effect of the consortium on growth of *L. monocytogenes* in cheeses

The statistical analysis (ANOVA) of the *L. monocytogenes* counts measured at the surface of control and assay Saint-Nectaire cheeses from ripening day 1 to 28 is reported in Table 3.

Table 3 Comparison of *Listeria* counts at the surface of all cheeses ripened at INRA with or without inoculation of microbial consortium AB in milk. Analysis of variance (ANOVA) using *Listeria* counts performed at the surface of cheeses.

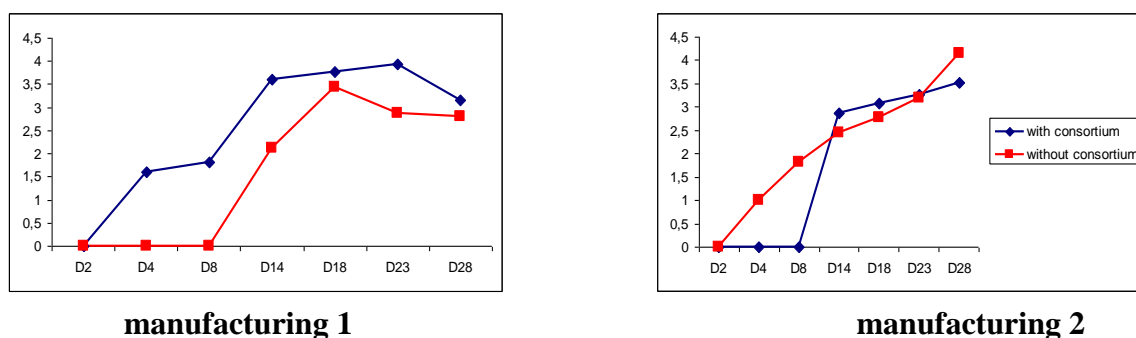
* mean values of *Listeria* counts in core and on the surface of cheeses ripened at INRA at different times (1, 8, 18, 28 days).

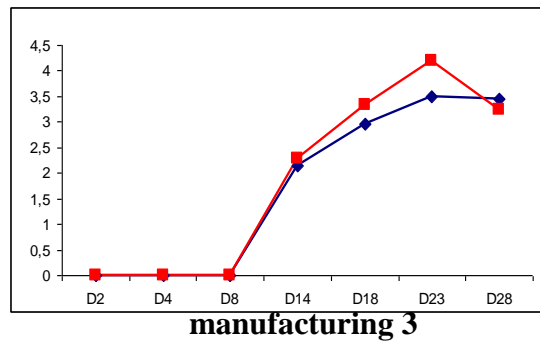
^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann Keul's statistical test ($P < 0.05$), with $a < b$.

Effect of consortium	List 2	list4	list8	list14	list18	list23	list28
Assays FE	presence	0,54 +/- 0.54	0,61 +/- 0.61	2,87 +/- 0.42	3,27 +/- 0.25	3,56 +/- 0.19	3,37 +/- 0.10
Controls FC	presence	0,33 +/- 0.33	0,61 +/- 0.61	2,28 +/- 0.10	3,18 +/- 0.2	3,42 +/- 0.4	3,40 +/- 0.4
Effect of day of manufacturing							
Manufacturing 1	presence	0.80 +/- 0.80	0.91 +/- 0.91	2.86 +/- 0.74	3.60 +/- 0.17	3.40 +/- 0.52	2.98 +/- 0.18
Manufacturing 2	presence	0.50 +/- 0.5	0.91 +/- 0.91	2.67 +/- 0.21	2.93 +/- 0.15	3.23 +/- 0.03	3.83 +/- 0.32
Manufacturing 3	presence	0.00	0.00	2.21 +/- 0.07	3.15 +/- 0.18	3.85 +/- 0.35	3.35 +/- 0.10

As shown in table 3, there was no significant effect of the consortium on the growth of *L. monocytogenes*. But the growth was dependent on the day of manufacturing as shown in table 3 and fig 2. Indeed, for manufacturing 1, the lag time was longer without consortium than with consortium and the *L. monocytogenes* count was lower without consortium. At the opposite, the lag phase was longer, but the *L. monocytogenes* count lower, with consortium in manufacturing 2. For manufacturing 3, the lag phase was similar in cheeses with or without consortium but the growth of *L. monocytogenes* was lower in cheeses with consortium.

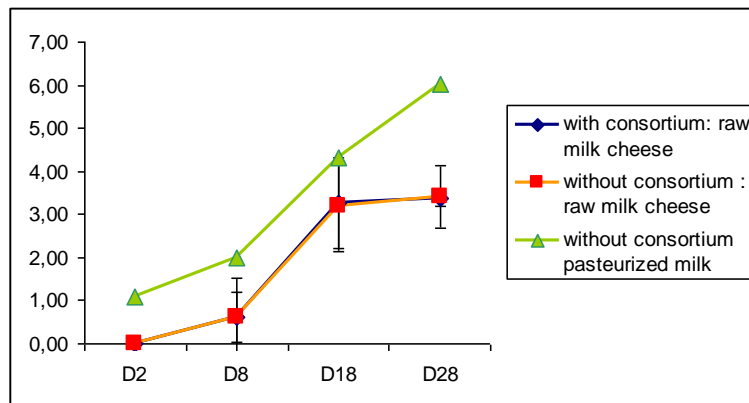
Fig 2 : Evolution of *Listeria monocytogenes* at the surface of raw milk cheeses prepared with or without consortium





These results can be compared with those previously obtained in cheeses prepared with pasteurized milk in WP2A (fig 3).

Fig 3 : Comparison of *L. monocytogenes* growth at the surface of raw milk cheeses and at the surface of pasteurised milk cheeses with commercial starter



Without addition of the consortium, the growth of *L. monocytogenes* was lower in raw milk cheeses than in pasteurized cheeses. This may indicate that the raw milk used for making St-Nectaire cheeses in this farm has some inhibitory properties similar to those of the consortium.

2.2 Effect of the consortium on pH, DM and a_w in cheeses

The statistical analysis (ANOVA) of the pH, DM and a_w measured in the core and on the surface of control and assay Saint Nectaire cheeses at each ripening day (2 to 28) is reported in Table 4.

In the core, the DM and a_w were not significantly affected by the inoculation of the consortium. In the same way, the pH was not affected by the inoculation excepted at 28 days of ripening where it was lower (average of 5.08) in assay cheese than in control cheese (average of 5.37). However, the pH and DM values were higher in cheeses from manufacturing 1 than those obtained from manufacturing 2 and 3.

At the surface, the pH, DM and a_w were not significantly affected by the inoculation of the consortium. But, throughout all ripening, pH at the surface of cheeses from manufacturing 1 were lower of 0.4 to 0.8 units than in those of manufacturing 2 and 3. In the same way, DM values varied according to the manufacturing and were lower in cheeses from manufacturing 3 until 8 days and lower in cheeses from manufacturing 2 at 18 and 28 days of ripening.

2.3 Effect of the consortium on microbial counts of cheeses

The statistical analysis (ANOVA) of the microbial population counts from ripening day 1 to 28 determined in the core and on the surface of control and assay Saint Nectaire cheeses (ripened at INRA and Lanobre) is reported in table 5 .

In the cheese core (Table 5a), the assay cheeses had higher count of lactic acid bacteria as *leuconostocs*, *enterococci* and *lactobacilli* and lower count of Gram negative bacteria. The counts of Gram positive catalase positive bacteria and yeasts were similar in the assay and control cheeses.

Cheeses from manufacturing 2 were distinguishable from those of manufacturing 1 and 3 by lower count of *leuconostocs* and higher count of *Enterococci*, Gram negative bacteria and yeasts at 28 days of ripening.

At the surface of cheeses (table 5b), the assay cheeses had a significantly lower count of Gram negative bacteria but higher count of lactic acid bacteria (*Lactobacillus* spp., *Leuconostoc* spp.) and Gram positive catalase positive bacteria but the differences were not significant. At the surface, the yeasts counts were higher in control than in assay cheeses at day 2 and 28. They were similar in both cheeses at days 8 and 18. Cheeses from manufacturing 1 were distinguishable from those of manufacturing 2 and 3 by highest counts of the *lactobacilli* and Gram negative bacteria, Gram positive catalase positive bacteria and yeasts.

2.4 Effect of the consortium on sugar and organic acid contents of cheeses

Lactose, galactose, glucose and organic acids (lactic acid, acetic acid) contents were measured in the core and the surface of all cheeses (controls and assays) at 2, 8, 18 and 28 days of ripening and statistical analysis (ANOVA) of these data are reported in Table 6.

a) Sugars (table 6a)

In the core of cheeses, lactose was rapidly depleted as it was only 1.6 g/kg at day 2. Throughout the ripening, it was still consumed in assay cheeses whereas there was no change in lactose content in control cheese. Until 18 days, galactose was more consumed in control cheeses than in assay cheeses and after 18 days the consumption was higher in assay cheeses. Galactose content decreased more in assay cheeses from manufacturing 1.

After 2 days, the lactose content was higher at the surface than in the core of cheeses and especially in the control cheeses. Throughout the ripening, the content was similar. At the cheese surface, the galactose content was lower than in the core. It was more consumed in assay cheeses than in control cheeses.

b) Acids (table 6b)

In the cheese core, the statistical analysis indicates that the lactic and acetic acid contents at 8, 18 and 28 days were significantly higher in assay cheeses than in control ones. Lactic acid contents increased until 28 days of ripening in assay cheeses whereas it decreased in control cheeses since 8 days of ripening. Acetic acid contents increased until 28 days of ripening in assay and control cheeses. There was no significant differences between the 3 manufacturing for the lactic acid production but the acetic acid production at 28 days was significantly lower in cheeses from manufacturing 2.

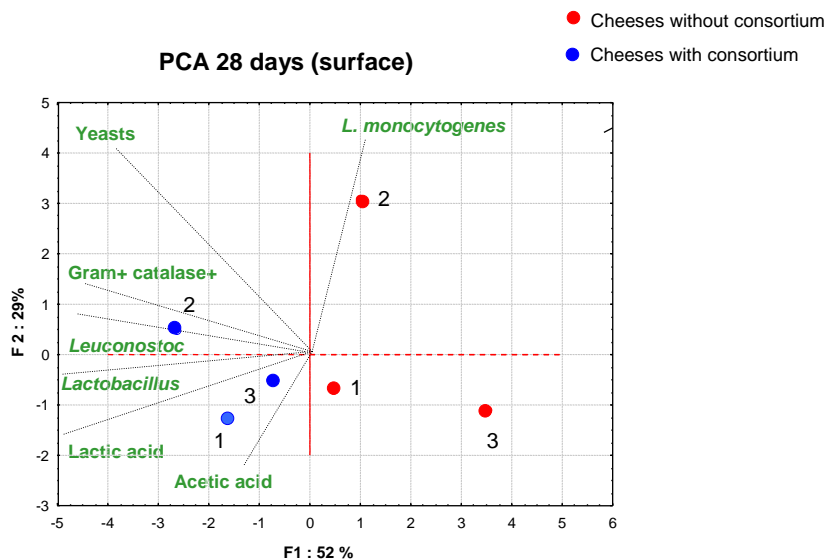
At the cheese surface, the lactic acid contents at 18 and 28 days were more important in assay cheeses than in control cheeses. Acetate content evolution was different at the surface of

cheeses than in the core. At the beginning of ripening acetate content was higher in the assay cheese than in the control ones. But, between 18d and 28 days, it increased more in control cheeses than in assay cheeses. There was no significant difference of lactic acid contents according to the manufacturing; while, acetic acid contents were higher in manufacturing 2 at 18 and 28 days.

2.5 Correlations *L. monocytogenes* – microbiological and biochemical data

A Principal Component Analysis (PCA) (fig 3) was performed with *L. monocytogenes* counts, microbiological and biochemical data showing significant differences between assay and control cheese surfaces at 28 days (cf ANOVA results of tables 3 to 6).

Fig 3 : Principal component analysis of the discriminate microbial and biochemical analysis at the surface of 28 days old cheeses. Attribute circle correlation and plot of assay (cheeses with consortium) and control (cheeses without consortium) cheeses (3 manufacturing).



The variables taken into account explained more than 80% of variability. Surface cheeses with consortium (assay) or without (control) were mainly differentiated on axis 1 (52%). Cheeses with consortium had the highest count of lactobacilli, leuconostocs and Gram positive catalase positive bacteria, the lowest count in yeasts and higher content in lactic acid. Cheeses from manufacturing 2, particularly control, had the highest counts of *L. monocytogenes* and yeasts, and the lowest content of acetic acid.

At 28 days of ripening on the surface, the *L. monocytogenes* counts were negatively correlated to acetic acid content ($r = -0.55$) and positively correlated to yeasts ($r = 0.74$). In the same way, at the surface, lactate contents were positively correlated with *Lactobacillus* spp. ($r = 0.93$) and Gram positive catalase positive bacteria counts ($r = 0.74$) at 28 days of ripening.

Table 4. Comparison of pH, DM and Aw in the core and at the surface of all cheeses ripened at INRA with or without inoculation of microbial consortium AB in milk. Analysis of variance (ANOVA) using pH, DM and a_w measurements performed in the core and at the surface of cheeses.

* mean values of pH, DM, Aw in core and on the surface of cheeses ripened at INRA at different times (1, 8, 18, 28 days).

^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann Keul's statistical test (P<0.05), with a<b.

a) Core												
<i>Effect of consortium</i>	pH 2	pH8	pH18	pH28	DM 2	DM8	DM18	DM28	Aw2	Aw8	Aw18	Aw28
Assays FE	5.52+/-0.22	5.29+/-0.02	5.35+/-0.07	5.08+/- 0.02 a	52.23+/-1.11	52.19+/-1.09	52.85+/-1.39	54.42+/-0.94	0.971+/-0.003	0.970+/-0.003	0.968+/-0.0	0.965+/-0.003
Controls FC	5.38+/-0.06	5.33+/-0.03	5.35+/-0.06	5.37+/-0.07 b	52.43+/-1.27	53.41+/-2.15	51.86+/-0.37	53.25+/-2.14	0.969+/-0.004	0.970+/-0.003	0.970+/-0.001	0.970+/-0.003
<i>Effect of day of ripening</i>												
Manufacturing 1	5.65+/-0.31	5.34+/-0.04	5.31+/-0.08	5.32+/-0.19	52.94+/-0.08	55.85+/-1.85	53.62+/-1.39	56.05+/-0.17	0.968+/-0.008	0.969+/-0.003	0.969+/-0	0.971+/-0.002
Manufacturing 2	5.40+/-0.1	5.28+/-0.03	5.36+/-0.12	5.17+/-0.11	50.76+/-0.76	50.88+/-0.65	51.24+/-2.0	51.03+/-2.03	0.970+/-0.005	0.970+/-0.003	0.970+/-0.002	0.969+/-0.003
Manufacturing 3	5.30+/-0.01	5.30+/-0.01	5.39+/-0.05	5.18+/-0.13	54.79+/-0.52	51.67+/-0.67	52.21+/-1.10	54.44+/-0.44	0.972+/-0	0.970+/-0.005	0.968+/-0.0	0.963+/-0.003
b) Surface												
<i>Effect of consortium</i>												
Assays FE	5.38+/-0.04	5.63+/-0.19	7.11+/-0.32	7.35+/- 0.08	54.01+/-1.28	54.14+/-2.54	63.16+/-4.89	67.36+/-1.94	0.969+/-0.003	0.973+/-0.002	0.960+/-0.02	0.968+/-0.002
Controls FC	5.48+/-0.11	5.75+/-0.37	7.35+/-0.08	7.34+/-0.1	55.0+/-1.81	53.18+/-1.41	65.70+/-2.77	68.84+/-1.59	0.955+/-0.009	0.973+/-0.002	0.965+/-0.009	0.973+/-0.004
<i>Effect of day of ripening</i>												
Manufacturing 1	5.34+/-0.04	5.34+/-0.04	6.53+/-0.03	5.94+/-0.44	55.51+/-1.99	57.50+/-1.5	70.65+/-0.05	69.32+/-0.32	0.968+/-0.005	0.973+/-0.001	0.939+/-0.009	0.971+/-0.002
Manufacturing 2	5.56+/-0.14	6.06+/-0.45	7.30+/-0.2	7.23+/-0.02	56.22+/-0.22	58.99+/-0.57	57.50+/-3.5	64.75+/-1.25	0.959+/-0.015	0.973+/-0.003	0.974+/-0.002	0.976+/-0.005
Manufacturing 3	5.39+/-0.031	5.68+/-0.28	7.25+/-0.3	7.34+/-0.13	51.80+/-0.3	52.50+/-0.51	65.15+/-0.35	70.23+/-1.28	0.958+/-0.011	0.974+/-0.003	0.975+/-0.001	0.965+/-0.001

Table 5. Comparison of microbial counts in the core and at the surface of cheeses ripened at INRA with or without inoculation of microbial consortium AB in milk and between 3 manufacturing trial. Analysis of variance (ANOVA) using microbial counts performed on different media described in 1.1.3 in the core and at the surface of cheeses.

^a mean values of microbial counts in core and on the surface of cheeses ripened at INRA at different times (1, 8, 18, 28 days).

^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann Keul's statistical test (P<0.05), with a<b.

a) core	MSE2	MSE8	MSE18	MSE28	FH2	FH8	FH18	FH28	SB2	SB8	SB18	SB28
<i>Effect of consortium</i>												
Assays FE	5.67+/-0.58 b	7.82+/-0.21 b	8.18+/-0.15 b	7.75+/-0.08 b	5.53+/-0.44 b	7.38+/-0.15 b	8.29+/-0.17 b	8.5+/-0.05	3.29+/-0.02 b	3.90+/-0.15	4.46+/-0.93	5.64+/-0.65
Controls FC	3.54+/-0.12 a	5.83+/-0.78 a	7.00+/-0.44 a	6.61+/-0.41 a	3.02+/-0.4 a	4.08+/-0.23 a	4.72+/-0.29 a	7.38+/-0.44	2.67+/-0.12 a	3.53+/-0.39	4.38+/-0.61	4.80+/-1.24
<i>Effect of day of manufacturing</i>												
Manufacturing 1	5.08+/-1.70	7.31+/-0.92	7.69+/-0.79	7.11+/-0.78	4.73+/-1.66	5.98+/-1.68	6.47+/-2.17	7.69+/-0.91	2.90+/-0.40	3.55+/-0.55	4.80+/-1.50	4.73+/-1.43
Manufacturing 2	4.44+/-0.97	5.95+/-0.64	7.15+/-0.85	6.90+/-0.82	4.32+/-0.43	5.81+/-1.48	6.34+/-1.76	8.37+/-0.13	2.93+/-0.33	3.65+/-0.34	4.36+/-1.06	6.84+/-0.42
Manufacturing 3	4.30+/-0.52	7.21+/-0.41	7.95+/-0.13	7.52+/-0.10	4.77+/-1.47	5.40+/-1.78	6.71+/-1.43	7.77+/-0.64	3.10+/-0.33	3.94+/-0.34	4.09+/-0.31	4.09+/-0.27
	PCAI2	PCAI8	PCAI18	PCAI28	CRBM2	CRBM8	CRBM18	CRBM28	OGA2	OGA8	OGA18	OGA28
<i>Effect of consortium</i>												
Assays FE	2.86+/-0.35 a	4.46+/-0.59	3.12+/-1.34 a	4.10+/-2.40 a	4.04+/-0.21	4.85+/-0.33	5.22+/-0.58	4.59+/-0.85	3.07+/-0.30	5.67+/-0.43	4.36+/-0.52	5.19+/-1.61
Controls FC	4.08+/-0.74 b	4.09+/-0.79	5.26+/-0.40 b	5.05+/-0.03 b	3.86+/-0.41	4.97+/-0.34	4.95+/-0.25	4.24+/-0.26	3.92+/-0.69	5.48+/-0.33	4.36+/-0.74	5.19+/-0.52
<i>Effect of day of manufacturing</i>												
Manufacturing 1	3.69+/-0.19	4.66+/-0.66	2.48+/-1.98 a	4.11+/-0.97 b	4.09+/-0.16	5.20+/-0.3	5.44+/-0.78	5.45+/-0.75 a	3.20+/-0.42	5.64+/-0.44	3.54+/-0.24 a	4.74+/-1.46 a
Manufacturing 2	2.60+/-0.3	3.76+/-0.99	5.26+/-0.36 c	6.85+/-1.79 c	4.08+/-0.47	4.46+/-0.04	4.84+/-0.61	3.77+/-0.47 b	3.41+/-0.41	5.62+/-0.61	3.95+/-0.05 a	6.41+/-1.97 b
Manufacturing 3	4.11+/-1.33	4.42+/-1.09	4.42+/-0.87 b	2.75+/-2.75 a	3.70+/-0.55	5.07+/-0.53	4.96+/-0.24	4.02+/-0.63 b	3.88+/-1.28	5.47+/-0.67	5.58+/-0.19 b	4.42+/-0.52 a

b) surface	MSE2*	MSE8	MSE18	MSE28	FH2*	FH8	FH18	FH28	SB2*	SB8	SB18	SB28
<i>Effect of consortium</i>												
Assays FE	6.60+/-0.35	8.24+/-0.14	8.18+/-0.13	7.82+/-0.08	5.07+/-0.11	7.21+/-0.32 b	8.09+/-0.12 b	8.46+/-0.07 b	3.76+/-0.36	5.11+/-0.41	6.72+/-0.26	6.16+/-1.29
Controls FC	4.60+/-0.33	7.57+/-0.28	8.22+/-0.09	6.67+/-0.17	1.1+/-0.6	4.31+/-0.54 a	6.09+/-0.26 a	7.15+/-0.29 a	3.68+/-0.75	4.90+/-0.86	7.03+/-0.37	7.52+/-0.2
<i>Effect of day of manufacturing</i>												
Manufacturing 1	5.97+/-1.33	7.92+/-0.21	8.30+/-0.006	7.88+/-0.08	3.72+/-1.43	6.22+/-1.60	7.41+/-0.81	7.99+/-0.42	2.75+/-0.35	3.85+/-0.65	6.25+/-0.05	7.40+/-0.26
Manufacturing 2	5.15+/-1.15	7.56+/-0.52	8.17+/-0.14	7.77+/-0.18	2.68+/-2.18	5.90+/-0.84	7.05+/-1.15	7.94+/-0.66	2.99+/-0.36	5.72+/-0.17	7.10+/-0.2	5.72+/-2.12
Manufacturing 3	5.68+/-0.52	8.24+/-0.27	8.12+/-0.2	7.54+/-0.16	2.86+/-2.36	5.17+/-1.92	6.81+/-1.04	7.49+/-0.89	4.43+/-0.58	5.44+/-0.51	7.26+/-0.21	7.40+/-0.18
	PCAI2*	PCAI8	PCAI18	PCAI28	CRBM2*	CRBM8	CRBM18	CRBM28	OGA2*	OGA8	OGA18	OGA28
<i>Effect of consortium</i>												
Assays FE	3.66+/-0.57	6.63+/-0.60	7.85+/-0.54	5.92+/-1.53	5.50+/-0.70	7.81+/-0.42	7.70+/-0.55	8.28+/-0.27	3.93+/-0.29	8.21+/-0.23	8.24+/-0.15	6.76+/-1.32
Controls FC	4.48+/-1.16	7.17+/-0.04	8.80+/-0.22	8.32+/-0.52	4.80+/-0.37	7.21+/-0.51	7.87+/-0.29	7.06+/-0.77	4.90+/-0.52	8.41+/-0.17	8.54+/-0.21	7.81+/-0.30
<i>Effect of day of manufacturing</i>												
Manufacturing 1	4.40+/-0.16	7.23+/-0.006	8.31+/-0.82	8.10+/-0.10	5.70+/-1.01	8.10+/-0.07	7.95+/-0.46	8.39+/-0.19	4.60+/-0.17	8.36+/-0.18	8.55+/-0.40	7.86+/-0.23
Manufacturing 2	3.21+/-0.60	7.17+/-0.06	8.64+/-0.27	7.17+/-3.17	4.25+/-0.01	7.15+/-0.17	7.54+/-0.9	7.95+/-0.56	4.00+/-0.08	8.54+/-0.21	8.39+/-0.13	6.25+/-2.14
Manufacturing 3	4.60+/-2.0	7.30+/-0.87	8.02+/-0.87	7.08+/-0.53	5.50+/-0.01	7.29+/-1.0	7.88+/-0.2	6.68+/-1.08	4.65+/-1.22	8.04+/-0.26	8.23+/-0.18	7.74+/-0.33

* MSE = dextrane-producing *Leuconostocs*, FH = heterofermentative lactobacilli, SB= enterococci, PCAI = Gram negative bacteria, CRBM = Gram positive catalase positive bacteria, OGA= yeasts

Table 6. Comparison of sugar and acid contains in the core and at the surface of cheeses ripened at INRA with or without inoculation of microbial consortium AB in milk. Analysis of variance (ANOVA) using acid contains in the core and at the surface of cheeses.

* mean values of sugar and acid contains (g/Kg DM) in core and on the surface of cheeses ripened at INRA at different times (1, 8, 18, 28 days).

^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann Keul's statistical test (P<0.05), with a<b.

a) Sugars

sugars core	glucose2	glucose 8	glucose 18	glucose 28	Galactose 2	Galactose 8	Galactose 18	Galactose 28	lactose2	lactose 8	lactose 18	lactose 28
<i>Effect of consortium</i>												
Assays FE	0.12+/-0.05 a	0.5+/-0.15	0.8+/-0.3	0.7+/-0.3	13.8+/-0.3	12.1+/-0.4	4.7+/-0.6 a	0.8+/-0.3 a	1.3+/-0.07	0.8+/-0.1	0.5+/-0.1 a	0.2+/-0.1 a
Controls FC	0.41+/-0.14 b	0.5+/-0.16	0.6+/-0.2	0.8+/-0.3	9.4+/-2.5	8.7+/-2.6	7.6+/-2.5 b	5.4+/-1.7 b	1.6+/-0.2	1.8+/-0.3	1.3+/-0.2 b	1.1+/-0.2 b
<i>Effect of day of manufacturing</i>												
Manufacturing 1	0.0 a	0.2+/- 0.2	0.0+/-0.00 a	0.2+/-0.1 a	13.9+/-0.05	5.9+/-2.9	1.9+/-0.6	0.1+/-0.04	1.0+/-0.04 a	0.7+/-0.2	0.7+/-0.3	0.9+/-0.3
Manufacturing 2	0.4+/-0.42 b	0.6+/-0.2	0.4+/-0.1 a	0.2+/-0.1 a	8.1+/-3.7	13.0+/-3.1	9.1+/-2.5	2.3+/-2.1	1.8+/-0.3 b	1.8+/-0.6	0.8+/-0.2	0.5+/-0.3
Manufacturing 3	0.4+/-0.07 b	0.9+/-0.2	1.6+/-0.3 b	1.2+/-0.4 b	12.9+/-0.1	12.8+/-0.5	7.8+/-2.0	4.2+/-1.6	1.5+/-0.02 b	1.4+/-0.2	1.2+/-0.3	0.8+/-0.4
<i>sugars surface</i>												
<i>Effect of consortium</i>												
Assays FE	2.3+/-0.1	2.8+/-1.0	0.8+/-0.2	0.0+/-0.00	12.2+/-0.4	2.1+/-1.5	4.7+/-0.09 a	0.00+/-0.00	5.7+/-0.7	1.9+/-0.3	0.5+/-0.2	0.6+/-0.06
Controls FC	3.0+/-0.3	2.1+/-1.0	0.6+/-1.0	0.0+/-0.0	9.8+/-0.7	1.8+/-0.6	7.6+/-0.6 b	0.0+/-0.0	10.0+/-1.7	2.4+/-0.3	1.3+/-0.1	0.7+/-0.4
<i>Effect of day of manufacturing</i>												
Manufacturing 1	2.3+/-0.2	0.0+/-0.0	0.0+/-0.0	0.0+/-0.0	10.8+/-0.1	3.0+/-0.3	1.7+/-0.7	0.0+/-0.0	6.2+/-0.7	1.8+/-0.2 a	0.7+/-0.2	0.7+/-0.2
Manufacturing 2	3.0+/-0.4	0.9+/-0.7	0.4+/-0.4	0.0+/-0.0	10.1+/-1.5	0.8+/-0.6	9.1+/-0.4	0.0+/-0.0	8.9+/-3.1	1.8+/-0.3 a	0.8+/-0.2	0.3+/-0.2
Manufacturing 3	2.6+/-0.3	6.5+/-1.3	1.6+/-0.2	0.0+/-0.0	12.1+/-0.4	2.1+/-2.0	7.7+/-0.7	0.0+/-0.0	8.4+/-1.3	2.8+/-0.1 b	1.2+/-0.1	0.9+/-0.4

b)Acids

acids core	lactate 2	lactate 8	lactate 18	lactate 28	acetate 2	acetate 8	acetate 18	acetate 28
<i>Effect of consortium</i>								
Assays FE	23.0+/-0.3	28.1+/-0.6 b	29.8+/-0.7 b	33.1+/-1.1 b	1.9+/-0.04 a	4.7+/-0.5	6.2+/-0.2 b	8.8+/-0.4 b
Controls FC	23.4+/-0.9	20.7+/-0.9 a	23.4+/-0.9 a	20.5+/-1.7 a	2.4+/-0.1 b	2.4+/-0.5	4.7+/-0.4 a	6.2+/-0.7 a
<i>Effect of day of manufacturing</i>								
Manufacturing 1	23.6+/-0.8	22.8+/-1.5	25.7+/-1.7	29.6+/-2.8	2.3+/-0.3	4.4+/-1.0	5.2+/-0.5	8.0+/-0.8 b
Manufacturing 2	24.3+/-0.2	25.4+/-1.3	24.6+/-2.6	28.9+/-5.3	2.1+/-0.04	3.0+/-0.8	5.5+/-0.9	6.8+/-0.9 a
Manufacturing 3	21.7+/-0.6	25.1+/-0.3	22.8+/-3.2	27.3+/-3.6	2.1+/-0.3	3.3+/-0.3	5.8+/-0.7	9.0+/-0.6 c
acids surface								
<i>Effect of consortium</i>								
Assays FE	18.5+0.6	16.2+/-2.9	28.7+/-1.3 b	18.0+/-1.1 b	0.6+/-0.2	7.9+/-1.7	6.2+/-2.7	13.1+/-2.6
Controls FC	13.3+/-2.0	15.1+/-1.3	20.1+/-1.3 a	11.5+/-1.0 a	0.5+/-0.1	5.8+/-1.1	4.7+/-3.4	19.9+/-4.7
<i>Effect of day of manufacturing</i>								
Manufacturing 1	17.9+/-0.5	10.6+/-2.7	25.7+/-1.7	15.1+/-1.5	0.1+/-0.01 a	7.3+/-1.7	5.2+/-3.1	11.9+/-3.0
Manufacturing 2	13.9+/-3.7	17.3+/-2.1	24.6+/-2.7	14.5+/-2.3	0.7+/-0.02 b	8.6+/-2.4	5.5+/-5.2	23.0+/-6.8
Manufacturing 3	15.8+/-1.3	19.1+/-3.3	22.8+/-1.7	14.8+/-2.4	0.9+/-0.1 b	4.8+/-1.1	5.8+/-3.1	14.4+/-3.8

2.6 Monitoring microbial dynamic

The presence of the different species constituting the consortium was also determined in the core and on the surface of the assay and control cheeses..

The results of specific PCR and V3 and V2 SSCP analysis on DNA extracted from culture media (CRBM, FH, MSE) or cheeses are summarized in table 7.

Table 7: Microbial dynamic of consortium strain by molecular analysis of assay and control cheeses

Consortium species	Core				Surface			
	assay		control		assay		control	
	2d	18d	2d	18d	2d	18d	2d	18d
<i>Lactobacillus casei</i>	x	x	x	x	x	x	x	x
<i>Lactobacillus curvatus</i>	na	na	na	na	na	na	na	na
<i>Lactobacillus plantarum</i>	x	x	-	x	x	x	x	x
<i>Lactobacillus farciminis</i>	na	na	na	na	na	na	na	na
<i>Leuconostoc citreum</i>	x	x	x	x	x	-	x	x
<i>Leuconostoc pseudomesenteroides</i>	x	x	-	-	x	x	-	-
<i>Staphylococcus equorum</i>	x	x	x	x	x	x	x	x
<i>Arthrobacter nicotianae</i>	x	x	x	x	x	x	x	x
<i>Corynebacterium flavescens</i>	x	x	-	x	x	x	x	-
<i>Corynebacterium casei</i>	x	x	x	x	x	x	x	x
<i>Brevibacterium linens</i>	-	-	-	-	-	x	x	-
<i>Brachybacterium rhamnosum</i>	-	x	-	x	-	x	x	-
<i>Macroccoccus caseolyticus</i>	x	-	-	-	-	x	-	-

X : the species was detected by SSCP analysis or by specific PCR analysis of different media (FH, MSE, CRBM) or cheeses .

- : the species was detected neither by SSCP on culture media or directly on cheeses, neither by PCR

na = not analyzed

From the results of table 7, it can be concluded that the following consortium species: *Lb. casei*, *Ln. citreum*, *Lb. plantarum* *C. flavescens*, *A. nicotianae*, *S. equorum*, *Brachy. rhamnosum*, *C. casei* were present both in the core and at the surface of assay and control cheeses. But *M. caseolyticus* and *Ln. pseudomesenteroides* were only detected in the assay cheeses at the surface or in the core.

The SSCP analysis of CRBM medium showed that the SSCP bacterial profiles on this medium inoculated with the surface of the control or assay cheeses at 2 and 18 days were both dominated by *A. nicotianae* and a peak coeluting with *Lb. casei* / *Ln. citreum* (cf figure 4). When the cheese cores were analysed, the SSCP profiles of CRBM medium of control and assay were different and richer than the surface. The SSCP profile of CRBM with assay cheeses were dominated by *A. nicotianae* and peaks of *S. equorum* , *M. caseolyticus*, *C. flavescens* . On the assay cheese SSCP profiles , if *A. nicotianae* was dominant at day 2, after 18 days *C. casei* and *C. flavescens* were the dominant peaks. On the SSCP profiles of CRBM medium after analysis of the control and assay cheeses, some unidentified peaks were observed.

The results confirmed that medium CRBM (Denis et al, 2001) is a suitable medium to study the implantation of Gram positive catalase positive bacteria even if some lactic acid bacteria can grow on it.

The DNA cheeses analysis by SSCP region V2 reveal that the profiles were dominated by *C. flavescens* and *Ln. citreum* . Some unidentified peaks were also detected.

Figure 4 : Analysis of bacteria growing on CRBM medium by SSCP region V3 : dominant peaks on the profiles

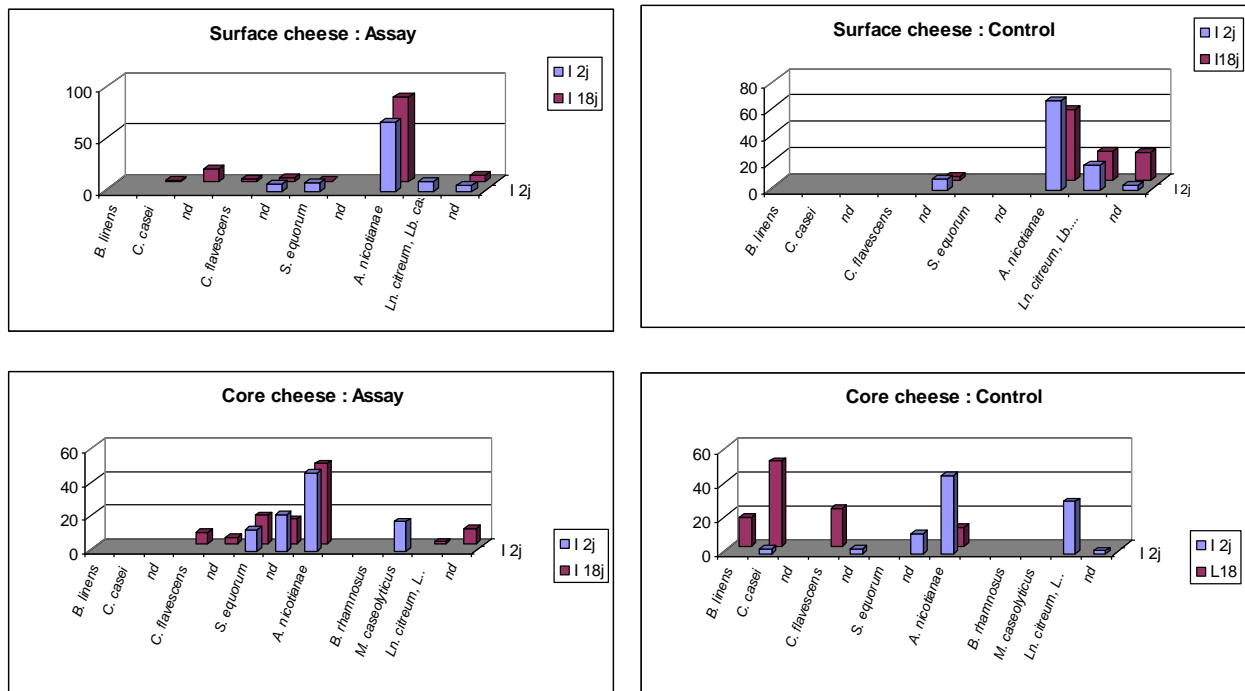
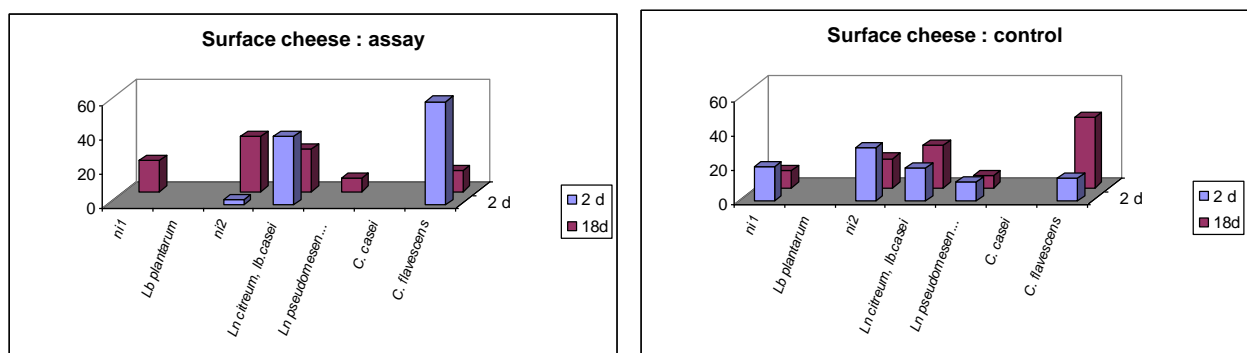


Figure 5 : analysis of total DNA from cheeses SSCP by SSCP region V2 : dominant peaks on the profiles



2.7 Effect of consortium on sensorial characteristics of cheeses

The effect of the microbial consortia on the sensorial characteristics (visual aspect, texture, odour, taste, aroma) of cheeses ripened at Lanobre during 28 days was analyzed by ANOVA. The results are presented in table 8.

Concerning the visual aspect of cheeses, significant differences between assay and control cheeses were only observed for opening hole which was higher in the core of assay cheeses (table 8a). However, an effect of the day of manufacturing was significant on the repartition of gray mould at the surface with scores lower in cheeses from manufacturing 3 and higher in cheeses from manufacturing 2. In the same way, the scores of core colour were lower for cheeses from manufacturing 1.

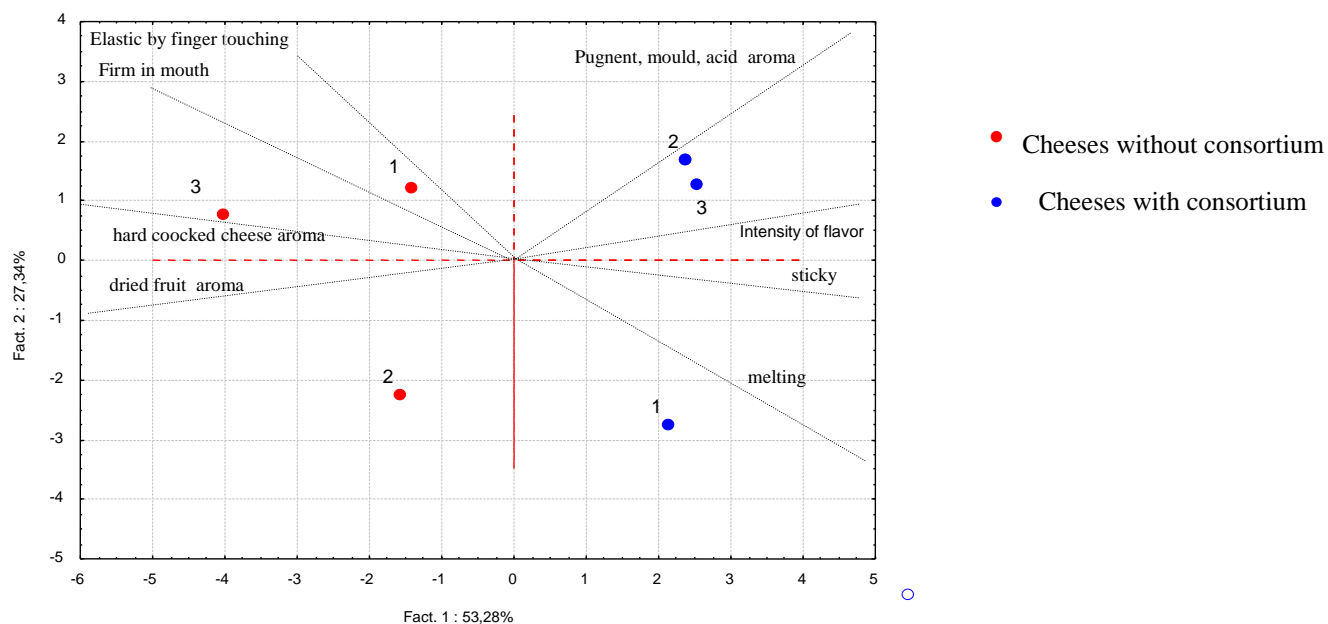
The inoculation of the consortium had a significant effect on the cheese texture (table 8b). The assay cheeses were significantly more elastic by finger touching and more sticky and melting in mouth than the control cheeses. On the contrary, the control cheeses were firmer in mouth than the assay one. There was no effect of the day of manufacturing on texture scores between control and assay cheeses.

Among the 10 odour descriptors (table 8c), only 2 descriptors had scores significantly different between the assay and the control cheeses. Butter and pungent odour scores were more intense in the assay than in the control. The intensity of these descriptors was higher in cheeses from manufacturing 1 whereas the hard cooked cheese scores were higher in cheeses from manufacturing 1 and 2.

The taste and aroma of cheeses were also affected by the inoculation of the consortium. Assay cheeses had higher intensity and saltiness, acid taste and mouldy aroma scores than control cheeses. Hard cooked cheese and dried fruit aroma were more intense in control cheeses than in assay ones.

A Principal Component Analysis (PCA) (fig 6) between assay and control cheeses was performed on sensory data measured which were significantly different at 28d (table 8).

Figure 6 : Principal component analysis of the discriminate sensory analysis at 28 days. Attribute circle correlation and plot of assay (FE) and control (FC) cheeses (3 manufacturing).



The first 2 components accounted for 80.6% of the overall variation. The plotting of individual on principal axis 1 and 2 produced an overall cluster for cheeses with and without consortium for the three manufacturings. Cheeses with consortium had the highest scores for general intensity and pungent, acid and mouldy aroma and were more sticky and melting than those without consortium. Cheeses without consortium were characterized by higher scores of hard cooked cheese and dried fruit aromas and were firmer in mouth, more elastic by finger touching.

Table 8. Comparison of sensorial descriptors of cheeses ripened at Lanobre with or without inoculation of microbial consortium AB in milk and between 3 days of manufacturing. Analysis of variance (ANOVA one factor) using sensorial data of cheeses.

^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann

Keul's statistical test (P<0.05), with a<b.

*mean values of sensorial data of cheeses ripened at Lanobre (3 repetitions)

a) visual aspect	Orange background	Quantity gray moulds	Repartition gray moulds	Quantity white mould	Repartition white moulds	Color of core	Quantity opening	Repartition opening		
<i>Effect of consortium</i>										
Assay	4.93 +/-0.38	3.92 +/-0.76	4.19 +/-0.68	6.50 +/-0.28	5.86 +/-0.57	6.00 +/-0.56	2.96 +/-0.6 b	4.07 +/-0.86		
Control	4.85 +/-1.26	3.00 +/-1.3	4.0 +/-0.83	5.75 +/-0.64	6.15 +/-0.31	6.22 +/-0.35	1.12 +/-0.73 a	4.56 +/-2.82		
<i>Effect of day of manufacturing</i>										
Manufacturing 1	6.23	3.61	4.40 b	6.23 b	5.42	5.21 a	2.24	3.11		
Manufacturing 2	3.43	4.99	5.22 c	5.29 a	5.99	6.51 b	1.06	6.38		
Manufacturing 3	5.01	1.78	2.68 a	6.86 b	6.60	6.61 b	2.82	3.46		
b) Texture	By finger touching		In mouth							
	firm	elastic	firm	sticky	melting					
<i>Effect of consortium</i>										
Assay	4.8 +/-0.5	5.1 +/-0.3 a	4.2 +/-0.3 a	2.8 +/-0.1 b	4.7 +/-0.4 b					
Control	4.6 +/-0.5	5.5 +/-0.1 b	5.2 +/-0.4 b	1.5 +/-0.2 a	3.7 +/-0.3 a					
<i>Effect of day of manufacturing</i>										
Manufacturing 1	4.0 +/-0.3	5.1 +/-0.6	4.3 +/-0.7	2.2 +/-	4.6 +/-0.6					
Manufacturing 2	4.8 +/-0.7	5.2 +/-0.03	4.7 +/-0.03	2.4 +/-1	4.2 +/-0.5					
Manufacturing 3	5.3 +/-0.2	5.6 +/-0.1	5.1 +/-0.7	1.9 +/-	3.8 +/-0.8					
c) Odor	Intensity	milk	butter	Hard cooked cheeses	Dried fruit	hay	grass	Ripening cellar	rancy	pugnent
<i>Effect of consortium</i>										
Assay	5.5 +/-0.4	0.2 +/-0.04	0.3 +/-0.1 b	1.4 +/-0.1	0.4 +/-0.2	0.1 +/-0.1	0.3 +/-0.1	0.9 +/-0.1	0.3 +/-0.03	0.5 +/-0.1 b
Control	5.2 +/-0.2	0.2 +/-0.2	0 a	1.6 +/-0.2	0.5 +/-0.1	0.1 +/-0.03	0.1 +/-0.1	0.9 +/-0.1	0.1 +/-0.2	0.3 +/-0.1 a
<i>Effect of day of manufacturing</i>										
Manufacturing 1	5.9 +/-0.4 b	0.1 +/-0.1	0.1 +/-0.1	1.7 +/-0.2 b	0.4 +/-0.2	0	0.1	0.9 +/-0.1	0.4 +/-0.3	0.5 +/-0.1
Manufacturing 2	4.8 +/-0.1 a	0.1 +/-0.1	0.2 +/-0.2	1.7 +/-0.1 b	0.6 +/-0.2	0.2 +/-0.1	0.2 +/-0.2	0.9 +/-0.1	0.1 +/-0.1	0.3
Manufacturing 3	5.2 +/-0.03 a	0.4 +/-0.1	0.2 +/-0.1	1.2 a	0.4 +/-0.1	0.1 +/-0.1	0.3	0.9 +/-0.03	0.2 +/-0.2	0.3 +/-0.2
d) Taste and aroma	Intensity	salted	acid	bitter	Hard cooked cheese	Dried fruit	mould	rancy	pugnent	persistence
<i>Effect of consortium</i>										
Assay	5.6 +/-0.1 b	4.9 +/-0.1 b	1.5 +/-0.4 b	1.2 +/-0.2	0.9 +/-0.01 a	0.2 +/-0.2 b	0.4 +/-0.1 b	0.3 +/-0.2	1.3 +/-0.4	4.9 +/-0.4
Control	4.7 +/-0.4 a	4.6 +/-0.1 a	0.6 +/-0.2 a	0.9 +/-0.3	1.4 +/-0.1 b	0.5 +/-0.1 a	0.1 +/-0.1 a	0.03 +/-0.04	0.8 +/-0.3	4.5 +/-0.3
<i>Effect of day of manufacturing</i>										
Manufacturing 1	5.2 +/-0.3	4.6 +/-0.2	0.9 +/-0.1	1.0 +/-0.3	1.1 +/-0.2	0.6 +/-0.04	0.3 +/-0	0.4 +/-0.3	1.0 +/-0.4	4.4 +/-0.2
Manufacturing 2	5.5 +/-0.3	4.8 +/-0.1	1.1 +/-0.6	1.3 +/-0.1	1.1 +/-0.2	0.4 +/-0.3	0.3 +/-0.3	0	1.3 +/-0.6	5.2 +/-0.3
Manufacturing 3	4.7 +/-0.8	4.9 +/-0.3	1.1 +/-0.8	0.8 +/-0.6	1.2 +/-0.3	0.2 +/-0.2	0.3 +/-0.3	0.1 +/-0.1	0.8 +/-0.5	4.5 +/-0.6

2.8 Effect of ripening conditions on microbial and sensorial qualities of cheeses

The effect of ripening place (INRA or Lanobre) on pH, DM, a_w , microbial flora, sugar and acid contents in the core and at the surface of assay and control cheeses was also evaluated by ANOVA (principal effect).

2.8.1 Effect of ripening conditions on pH, DM and a_w evolution in cheeses

The statistical analysis (ANOVA) of the pH, DM and a_w data measured in the core and on the surface of control and assay at all ripening times at INRA and Lanobre is reported in Table 9.

Table 9. Comparison of pH, DM and Aw in the core and at the surface of control and assay cheeses ripened at INRA and Lanobre.

Analysis of variance (ANOVA) using pH, DM and Aw measurements performed in the core and at the surface of cheeses.

¹ mean values of pH, DM, Aw in core and on the surface of control cheeses at different times (1, 8, 18, 28 days). 3 repetitions

^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann Keul's statistical test ($P < 0.05$), with $a < b$.

a) Core	pH8	pH18	pH28	DM8	DM18	DM28	Aw8	Aw18	Aw28
<i>Effect of ripening</i>									
INRA	5.31±/0.02	5.35±/0.04	5.22±/0.07	52.8±/1.1	52.35±/0.68	53.83±/1.08	0.970±/0.002	0.969±/0.0	0.967±/0.001 b
Lanobre	5.34±/0.01	5.33±/0.05	5.28±/0.1	53.6±/0.3	53.11±/1.12	52.75±/0.69	0.965±/0.001	0.966±/0.001	0.960±/0.002 a
<i>Effect of day of manufacturing</i>									
Manufacturing 1	5.4±/0.01	5.31±/0.04	5.40±/0.10 b	54.6±/1.1	53.6±/0.8	54.9±/0.67 b	0.968±/0.001	0.969±/0.001	0.965±/0.003 b
Manufacturing 2	5.3±/0.03	5.37±/0.07	5.19±/0.10 a	52.0±/0.7	50.8±/0.6	51.0±/1.0 a	0.968±/0.002	0.967±/0.001	0.967±/0.001 b
Manufacturing 3	5.30±/0.01	5.34±/0.05	5.16±/0.10 a	53.0±/0.9	53.8±/1.2	54.0±/0.4 b	0.967±/0.003	0.966±/0.001	0.960±/0.002 a
b) Surface									
<i>Effect of ripening</i>									
INRA	5.69±/0.19	7.02±/0.17	7.17±/0.14	53.66±/1.32 a	64.43±/2.58	68.10±/1.17 b	0.973±/0.001	0.963±/0.008	0.971±/0.002
Lanobre	6.13±/0.33	6.67±/0.13	7.29±/0.02	58.6±/0.88 b	62.88±/3.18	61.86±/1.12 a	0.971±/0.002	0.974±/0.003	0.975±/0.003
<i>Effect of day of manufacturing</i>									
Manufacturing 1	5.4±/0.02 a	6.53±/0.02 a	7.09±/0.20	57.79±/1.35	70.65±/0.03 c	65.47±/2.42	0.974±/0.000	0.953±/0.009 a	0.972±/0.002 a
Manufacturing 2	5.6±/0.4 b	6.85±/0.26 b	7.25±/0.02	54.42±/2.02	55.71±/1.86 a	62.360±/1.52	0.974±/0.001	0.976±/0.001 b	0.979±/0.003 b
Manufacturing 3	5.8±/0.2 b	7.16±/0.13 c	7.34±/0.05	56.20±/2.15	64.79±/0.31 b	67.12±/2.04	0.969±/0.002	0.977±/0.003 b	0.967±/0.001 a

With or without inoculation of consortium or not, the pH, DM and a_w values in the cheese core were not significantly different between cheeses ripened at INRA and those ripened at Lanobre. Cheeses from manufacturing 1 had also higher pH values than those observed for the other manufacturing.

At the surface, there was no significant differences in pH, DM and a_w values according to the place of ripening. Cheeses from manufacturing 3 had the highest pH values.

2.8.2 Effect of ripening conditions on microbial counts in cheeses

The statistical analysis (ANOVA) of the microbial population counts at different times of ripening determined in the core and on the surface of control and assay cheeses ripened at INRA and Lanobre is reported in Table 10.

Table 10. Comparison of microbial counts in the core and at the surface of control and assay cheeses ripened at INRA and at lanobre. Analysis of variance (ANOVA) using microbial counts performed on different media described in 1.1.3 in the core and at the surface of cheeses.

¹ mean values microbial counts in core and on the surface of control cheeses at different times (1, 8, 18, 28 days). 3 repetitions.

^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann Keul's statistical test (P<0.05), with a<b.

a) Core	MSE 8	MSE 18	MSE 28	FH 8	FH 18	FH 28	SB 8	SB 18	SB 28
<i>Effect of ripening</i>									
INRA	6.82+/-0.6	7.60+/-0.34	7.18+/- 0.31	5.72+/-0.7	6.50+/-0.81	7.94+/-0.32	3.71+/-0.2	4.42+/-0.5	5.22+/-0.65
Lanobre	7.45+/-0.3	7.58+/-0.26	7.66+/-0.11	5.82+/-0.9	7.72+/-0.41	8.07+/-0.30	4.05+/-0.2	5.31+/-0.3	5.69+/-0.32
<i>Effect of day of manufacturing</i>									
Manufacturing 1	7.3+/-0.5	7.71+/-0.41	7.43+/-0.4	6.2+/-1.1 a	7.29+/-1.03	8.06+/-0.44	3.8+/-0.3	5.25+/-0.8	5.46+/-0.72
Manufacturing 2	7.0+/-0.9	7.39+/-0.48	7.18+/-0.4	5.7+/-1.0 b	7.05+/-0.9	8.09+/-0.39	3.7+/-0.2	5.14+/-0.6	6.14+/-0.56
Manufacturing 3	7.2+/-0.2	7.67+/-0.17	7.63+/-0.08	5.4+/-1.0 b	7.0+/-0.8	7.87+/-0.35	4.2+/-0.2	4.20+/-0.2	4.76+/-0.48
	PCAI 8	PCAI 18	PCAI 28	CRBM 8	CRBM 18	CRBM 28	OGA 8	OGA 18	OGA 28
<i>Effect of ripening</i>									
INRA	4.27+/-0.5	4.19+/-0.79	4.57+/- 1.1	4.91+/-0.21	5.08+/-0.29	4.41+/-0.41	5.57+/-0.24	4.36+/-0.40	5.19+/-0.76
Lanobre	3.12+/-0.8	4.80+/-0.23	5.01+/-0.7	5.09+/-0.3	5.52+/-0.27	6.23+/-0.68	4.79+/-0.19	4.63+/-0.14	5.04+/-0.49
<i>Effect of day of manufacturing</i>									
Manufacturing 1	4.43+/-0.3	3.70+/-1.08	4.19+/-0.41	5.6+/-0.3 c	5.71+/-0.44	5.81+/-0.32	5.4+/-0.2	4.03+/-0.3	4.51+/-0.6
Manufacturing 2	3.1+/-0.9	5.20+/-0.15	6.26+/-0.82	4.4+/-0.2 a	5.28+/-0.36	4.73+/-1.25	5.1+/-0.4	4.43+/-0.3	4.6776+/-0.9
Manufacturing 3	4.4+/-1.1	4.58+/-0.5	3.93+/-1.53	5.0+/-0.2 b	4.91+/-0.10	5.41+/-1.28	5.0+/-0.4	5.02+/-0.4	5.18+/-0.8
b) surface									
<i>Effect of ripening</i>									
INRA	7.91+/-0.21 b	8.20+/-0.07	7.73+/- 0.09 b	5.76+/-0.71	7.09+/-0.46 a	7.80+/-0.32	5.0+/-0.43 a	6.87+/-0.21	6.84+/-0.66
Lanobre	7.23+/-0.37 a	7.85+/-0.20	7.10+/-0.23 a	5.68+/-0.86	7.71+/-0.35 b	8.01+/-0.20	5.69+/-0.24 b	7.28+/-0.23	7.91+/-0.36
<i>Effect of day of manufacturing</i>									
Manufacturing 1	7.70+/-0.24	8.18+/-0.09	7.27+/-0.39	6.37+/-1.01	7.66+/-0.46 c	8.07+/-0.24	4.40+/-0.41 a	6.53+/-0.2 a	7.87+/-0.38
Manufacturing 2	7.26+/-0.58	8.10+/-0.22	7.74+/-0.10	5.59+/-0.9	7.45+/-0.58 b	8.10+/-0.32	5.90+/-0.14 b	7.49+/-0.25 b	7.01+/-1.14
Manufacturing 3	7.74+/-0.34	7.80+/-0.24	7.24+/-0.19	5.20+/-1.0	7.11+/-0.58 a	7.56+/-0.38	5.74+/-0.27 b	7.22+/-0.14 b	7.25+/-0.33
	PCAI 8	PCAI 18	PCAI 28	CRBM 8	CRBM 18	CRBM 28	OGA 8	OGA 18	OGA 28
<i>Effect of ripening</i>									
INRA	6.90+/-0.29	8.32+/-0.34	7.12+/- 0.90	7.51+/-0.32	7.79+/-0.28	7.67+/-0.45	8.31+/-0.14 b	8.39+/-0.13 b	7.28+/-0.65
Lanobre	6.07+/-0.78	8.30+/-0.26	7.61+/-0.69	7.80+/-0.20	8.44+/-0.22	8.49+/-0.84	7.67+/-0.3 a	8.01+/-0.06 a	7.49+/-0.46
<i>Effect of day of manufacturing</i>									
Manufacturing 1	7.15+/-0.04	8.52+/-0.41	8.05+/-0.21	8.17+/-0.15	8.07+/-0.30	8.96+/-0.34	8.21+/-0.13 b	8.27+/-0.24	7.69+/-0.14
Manufacturing 2	7.07+/-0.21	8.57+/-0.13	7.71+/-1.57	7.24+/-0.16	7.85+/-0.41	8.52+/-0.41	8.37+/-0.15 b	8.24+/-0.11	7.23+/-1.04
Manufacturing 3	5.22+/-1.04	7.86+/-0.40	6.34+/-0.48	7.55+/-0.44	8.43+/-0.33	6.76+/-1.13	7.39+/-0.4 a	8.09+/-0.11	7.23+/-0.66

MSE = *Leuconostoc* producing dextrane, FH = heterofermentative *Lactobacilli*, SB= *Enterococci*, PCAI = Gram negative bacteria, CRBM = Gram positive catalase positive bacteria, OGA= yeasts

In the cheese core, there was no significant effect of ripening place on counts on MSE, FH, SB, PCAI, CRBM and OGA media. Nevertheless, the level of Gram positive catalase positive bacteria growing on CRBM medium at 18 and 28 days was higher in cheeses ripened at Lanobre than in those ripened at INRA. Cheeses from manufacturing 1 had lower population levels at 8 days on FH medium and higher level of yeasts on OGA medium.

At the surface of cheeses, the level of leuconostocs on MSE medium at 8 and 28d and the yeast counts at 8 and 18 days were higher in cheeses ripened at INRA than in those ripened at lanobre. The level of lactobacilli on FH medium at 18d and *Enterococcus* spp. on SB medium at 8d, 18 and 28d were the highest in the cheeses ripened at Lanobre .

2.8.3 Effect of ripening conditions on sugars and organic acids contents in cheeses

The statistical analysis (ANOVA) of sugar and organic acids at all time of ripening determined in the core and on the surface of control or assay Saint Nectaire cheeses ripened at INRA and Lanobre is reported in Table 11.

Table 11 . Comparison of sugar and organic acids contents in the core and at the surface of control and assay cheeses ripened at INRA and Lanobre. Analysis of variance (ANOVA) using biochemical data in the core and at the surface of cheeses.

¹ mean values of contents in core and on the surface of cheeses at different times (1, 8, 18, 28 days). 3 repetitions.

^{a, b} letters indicate homogeneous statistical processing groups within column that were significantly different according to the Newmann Keul's statistical test ($P < 0.05$), with $a < b$.

Core	Lactose 8	Lactose 18	Lactose 28	Glucose 8	Glucose 18	Glucose 28	Galac 8	Galac 18	Galac 28
<i>Effect of ripening</i>									
INRA	13+/-0.4	0.93+/-0.2	0.66+/-0.2	0.5+/-0.2	0.86+/-0.3	0.71+/-0.4	10.6+/-2.0	6.18+/-1.8	3.10+/-1.0
Lanobre	0.96+/-0.2	0.84+/-0.2	0.77+/-0.3	0.19+/-0.1	0.4+/-0.2	0.3+/-0.1	10.26+/-2.0	3.63+/-1.8	1.31+/-1.6
<i>Effect of day of manufacturing</i>									
Manufacturing 1	0.85+/-0.2	1.02+/-0.3	0.85+/-0.3	0.17+/-0.2	0.10+/-0.06 a	0.15+/-0.09 a	9.1+/-2.9	1.42+/-0.6	0.08+/-0.04
Manufacturing 2	1.44+/-0.6	0.79+/-0.2	0.53+/-0.3	0.32+/-0.2	0.38+/-0.1 a	0.16+/-0.09 a	9.87+/-3.1	5.48+/-2.5	2.33+/-2.1
Manufacturing 3	1.1+/-0.2	0.84+/-0.3	0.77+/-0.4	0.55+/-0.2	1.13+/-0.3 b	1.20+/-0.4 b	12.3+/-0.5	7.85+/-2.0	4.21+/-1.6
surface									
<i>Effect of ripening</i>									
INRA	2.13+/-0.3	0.98+/-0.2	0.63+/-0.2	2.46+/-1.3	0.09+/-0.09	0 a	1.99+/-0.4	0.07+/-0.07 a	0.16+/-0.2 a
Lanobre	2.1+/-0.3	0.67+/-0.1	0.78+/-0.3	2.90+/-0.5	1.48+/-1.0	0.38+/-0.1 b	4.23+/-1.4	1.34+/-0.6 b	0.4+/-0.2 b
<i>Effect of day of manufacturing</i>									
Manufacturing 1	1.70+/-0.2 a	0.6+/-0.2	0.39+/-0.2	2.64+/-1.1	1.78+/-1.5	0.21+/-0.1	2.75+/-0.3	0.87+/-0.7	0.27+/-0.2
Manufacturing 2	1.77+/-0.3 a	1.03+/-0.2	0.57+/-0.2	1.07+/-0.7	0.37+/-0.3	0.12+/-0.1	1.54+/-0.6	0.42+/-0.4	0.38+/-0.4
Manufacturing 3	2.88+/-0.1 b	0.84+/-0.1	1.17+/-0.4	4.29+/-1.3	0.22+/-0.2	0.24+/-0.2	5.03+/-2.0	0.83+/-0.7	0.20+/-0.2

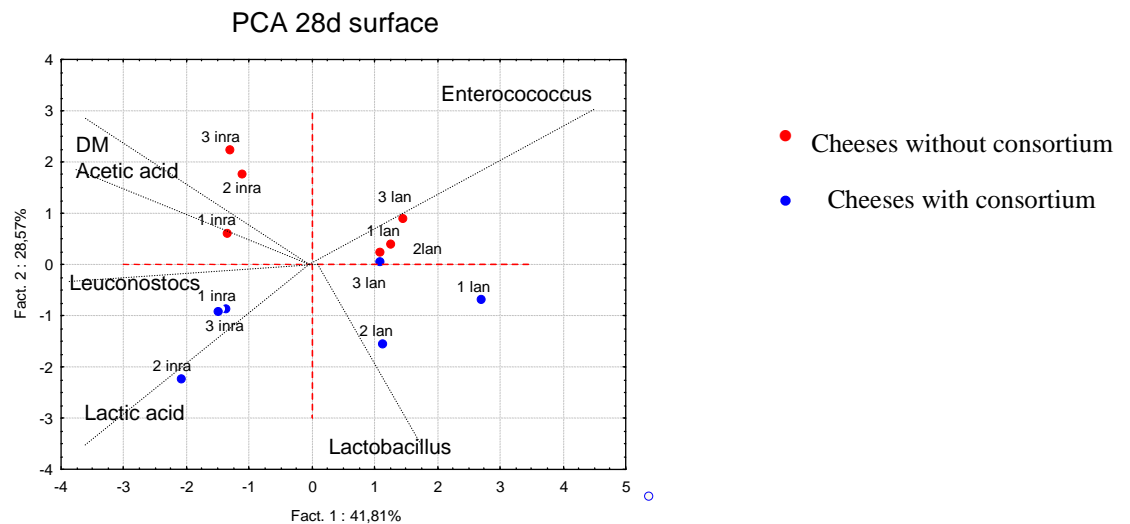
Core	Lactate 8	Lactate 18	Lactate 28	Acetate 8	Acetate 18	Acetate 28
<i>Effect of ripening</i>						
INRA	24.42+/-1.3	24.36+/-1.8	28.58+/-2.4	3.55+/-0.5	5.48+/-0.42	7.91+/-0.6
Lanobre	25.09+/-0.5	25.75+/-2.1	25.03+/-3.6	3.65+/-0.7	5.20+/-0.7	6.36+/-0.6
<i>Effect of day of manufacturing</i>						
Manufacturing 1	23.54+/-1.5	25.18+/-1.7	27.44+/-2.8	3.55+/-1.0	5.57+/-0.5	7.0+/-0.8 a
Manufacturing 2	25.81+/-1.3	26.0+/-2.6	26.96+/-5.3	3.81+/-0.8	4.8+/-0.9	6.16+/-0.9 a
Manufacturing 3	24.93+/-0.3	24.0+/-3.2	26.01+/-3.6	3.46+/-0.3	5.65+/-0.7	8.24+/-0.6 b
surface						
<i>Effect of ripening</i>						
INRA	15.66+/-2.9	15.75+/-1.9	14.77+/-1.5 b	6.87+/-1.0 b	14.80+/-1.9 b	16.46+/-2.9 b
Lanobre	18.29+/-1.3	13.73+/-1.3	11.26+/-1.4 a	1.20+/-0.2 a	4.97+/-2.6 a	1.71+/-0.1 a
<i>Effect of day of manufacturing</i>						
Manufacturing 1	14.11+/-2.7	14.2+/-1.7	13.45+/-1.5	4.38+/-1.7	10.81+/-3.1	6.82+/-3.0
Manufacturing 2	18.46+/-2.1	13.91+/-2.7	13.73+/-2.3	4.81+/-2.4	11.53+/-5.2	12.44+/-6.8
Manufacturing 3	18.37+/-3.3	16.1+/-1.7	11.86+/-2.4	2.93+/-1.1	7.17+/-3.1	8.0+/-3.8

In the core of cheeses, glucose, galactose, lactose, lactic and acetic acids contents were not significantly different according the ripening place. The cheeses from manufacturing 3 had the highest levels of glucose and galactose at 28d.

The surface of cheeses ripened at Lanobre presented higher glucose and galactose contents and lower acetic and lactic acid contents than those ripened at INRA.

A Principal Component Analysis (PCA) (fig 7) was performed with physico-chemical, microbial and biochemical data significantly different at 28 days (cf table 9, 10 and 11).

Fig 7 : Principal component analysis of the discriminate physico-chemical, microbial and biochemical data analysis at 28 days. Attribute circle correlation and plot of assay (FE) and control (FC) cheeses ripened at INRA and Lanobre (3 manufacturings).



The first 2 components accounted for 70% of the overall variation. By PCA analysis, cheeses were separated in 4 groups according to i) the place of ripening (INRA and Lanobre), ii) the consortium inoculation (with or without). The surface of 28 days old cheeses ripened at INRA were mainly characterized by higher level of leuconostocs and higher lactic and acid contents. Cheeses ripened at Lanobre had higher level of lactobacilli and enterococci.

B) Effect of the microbial consortium on the development of *Listeria monocytogenes* in Cantal cheeses.

To validate the efficiency of the consortium AB inoculated in milk, it was tested in Cantal cheese plant naturally contaminated with *Listeria*.

1 Methodology

1.1 Cheese making and strains inoculation

Cantal cheeses were prepared two times per day in a farm producing this kind of cheese. 3 control cheeses were manufactured one week and the 3 assay cheeses with the consortium AB were manufactured the following week.

Control cheeses were just inoculated with the usual commercial starter cultures daily used by the producer. Assay cheeses were supplemented with the anti-*Listeria* consortium.

The farm was selected as it has frequently a contamination of its cheeses by *L. monocytogenes*.

1.2 Cheese ripening conditions

One cheese of each manufacturing was transferred at INRA and ripened in an INRA cellar (3 months at 9°C and 98% hygrometry). The ripening cares were applied as usual for this type of cheese.

1.3 Analysis

L. monocytogenes was counted in the milk, after pressing of cheeses (6h), before moulding (1 days) during manufacturing, in the core at 8 and 18 days of ripening and in the core and at the surface at 28 days, 2.5 months and 3 months according to EN ISO/11290-2 by the accredited laboratory (Laboratoire Interprofessionnel du Massif Central LIAL, Aurillac, France).

2 Results

Counts of *L. monocytogenes* in cheeses

The presence- absence and the level of *L. monocytogenes* during manufacturing and ripening of control and assay cheeses are presented in table 12.

Table 12 : Presence and counts of *L. monocytogenes* in assay and control cheeses during manufacturing and ripening

- = absence of *L. mono*, + = presence of *L. mono*, ■ = no analysed

	Core						Surface					
	Cont 1	Cont 2	Cont 3	Assay 1	Assay 2	Assay 3	Cont 1	Cont 2	Cont 3	Assay 1	Assay 2	Assay 3
milk	-	-	-	-	-	-	■					
after pressing	-	+	-	-	+	-	■					
before moulding	+	-	+	+	+	+	■					
8 days	<10	<10	<10	<10	<10	<10	■					
18 days	<10	<10	<10	<10	<10	<10	■					
1 month	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
3 months	-	<10	-	-	-	-	-	<10	<10	-	-	-

L. monocytogenes was no detected in any milk used for either the assay or the control cheeses. It was sporadically detected in assay and control cheeses during the manufacturing whereas it was always present in the core of all cheeses at 8, 18 and 28 days. However, the level was low (<10cfu/g). At two months of ripening, *L. monocytogenes* was no more detected in assay cheeses (core and surface), while it was present in the core of control cheese C2 and at the surface of both control cheeses C2 and C3.

Conclusions

The addition of an anti-*Listeria* consortium (efficient in pasteurized milk) had no marked anti-*Listeria* effect in the raw milk cheeses manufactured in the farm selected for this study. The growth of *Listeria monocytogenes* and lag phase was different from a day of manufacturing to another. The addition of consortium delayed and reduce the growth of *L. monocytogenes* only in one experiment. The differences observed for the anti-*Listeria* consortium efficiency in raw and pasteurized milk cheeses may be explained by the fact that in pasteurized milk the inhibition was expressed in comparison with cheeses only prepared with *S. thermophilus*. By comparing the growth of *L. monocytogenes* in pasteurized milk with consortium and the raw milk cheese without consortium, the inhibition in raw milk cheese was inhibitory to the same extent than in pasteurized cheeses with the consortium.

The low or absence of inhibition can be also due to the fact that *L. monocytogenes* was inoculated at the surface whereas in pasteurized milk conditions, the highest inhibition was observed in the core of cheese after inoculation in cheeses .

The addition of the consortium led to a higher level of lactobacilli, Gram positive catalase positive bacteria but the species of the consortium were also found in raw milk cheese without consortium. It was not surprising as the consortium species were isolated from cow raw milk in the Saint-Nectaire area.

The inoculation of microbial consortium had an effect on sensorial properties of cheeses. Indeed, cheeses with consortium addition had the highest scores for general intensity and pungent, acid and mouldy aroma, less hard cooked cheese and dried fruit aromas and were more sticky and melting than those without consortium.

The study also confirmed that the microbial and physico-chemical characteristics of cheese was dependent on the ripening place. Some microbial flora developed more in industrial ripening at Lanobre. This had an incidence on pH, L-lactate and acetate production only in the core of cheese.

Test of this consortium in Cantal cheeses prepared in a farm presenting recurrent cheese contamination by *L. monocytogenes* gave some interesting results as *L. monocytogenes* was no more detected after 3 months in cheeses with consortium while the pathogen was still present in some samples without consortium.

C) Effect of microbial consortia on the development of *Listeria monocytogenes* Pont l'Evêque cheeses.

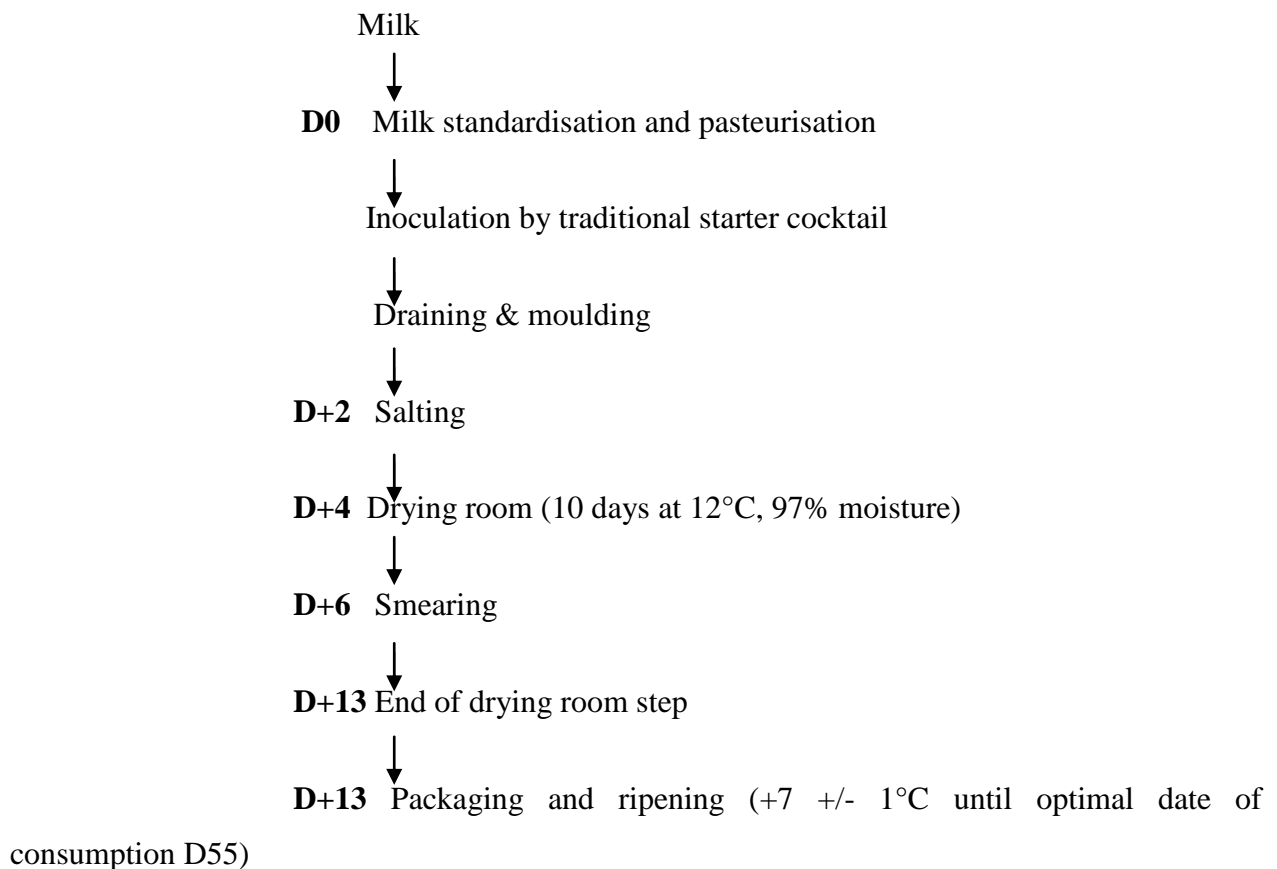
The objective of this task was to evaluate the impact of the developed anti-*Listeria* consortium in a different cheese technology: a smear-ripened cheese technology was chosen. In this context, the incidence of the consortium selected by INRA in terms of organoleptic impact, flora behaviour (including impact on inoculated *Listeria*) was studied on Pont l'Evêque cheeses, with (assay cheese) or without (control cheese) anti-*Listeria* consortium.

The assays performed on Pont-l'Evêque corresponded to a collaborative work between 2 ACTIA centres, ADRIA Normandie and ADRIA Développement.

1 Methodology

1.1 Cheese making and strains inoculation

Pont-l'Evêque production was performed during the month of June 2009, in pilot scale conditions, at the technological hall of the St Lô-Thère agricultural high school. Three cheesemaking dates, corresponding to 3 different milks, were chosen for the analysis performed during this study. The different steps of the cheesemaking process are presented in the following figure (Figure 8).



For each production period, a control (with only traditional starters) and a assay (traditional starters supplemented by the anti-*Listeria* consortium) were obtained for this cheese technology. For each condition (control and assay), a 60 litre vat was used allowing for the production of 20 cheeses.

During this study, in order to allow for a good implantation of *Listeria monocytogenes* during the artificial contaminations, salting was performed after 24h ; then half of each condition was transferred to ADRIA Développement for cheese surface inoculation by *L. monocytogenes* (5 CFU/cm²) and smearing with dehydrated Pont l'Evêque surface microflora according to provided protocols. The sample with (assay) or without(control) INRA consortium were then stored in the same conditions during the whole technological process.

The composition of the traditional starters used is presented in annex 1 while the microorganisms composing the anti-*Listeria* consortium are described in Table A1 (p3).

1.2 Microbiological and physico-chemical analyses

During this study, different microbiological and physico-chemical parameters (pH and Aw) were monitored at different cheesemaking dates: D+1, D+6, D+8, D+17, D+28, D+41 and D+55. The different microbial populations were enumerated on various media: FH (*Lactobacillus* spp.), MSE (*Leuconostoc* spp.), CRBM (Gram⁺ catalase⁺ surface flora), PCAi (Gram⁻), OGA (yeast). *L. monocytogenes* counts were performed on ALOA after smearing and along ripening at D+3, D+5, D+14, D+19, D+25, D+31, D+38 and D+53. Moreover, control cheese dedicated to sensorial analysis, *Listeria monocytogenes* contamination research were performed at the beginning of the tests and just before sensory evaluation tastings.

1.3 Molecular biology analysis

1.3.a Consortium monitoring by M13-PCR

- **Strain isolation**

From each enumeration performed on Pont-L'Evêque cheeses obtained using the INRA consortium, representatives colonies observed on the CRBM, MSE and FH were selected to determine if the consortium strain were able to implant in the cheese. A total, of 86 isolates were conserved for molecular biology characterization (M13-PCR typing and comparison to the genetic profiles obtained for the 14 strains constituting the INRA consortium).

- **Strain typing**

The 86 isolates were characterized using the M13-PCR method (Guinebretière and Nguyen-The 2003; Henderson et al. 1994) allowing for the generation of genetic profiles allowing for grouping and typing of the isolates. Each M13-PCR reaction was performed in the presence of 1 µl purified DNA, 2.0 µM M13 primer (5'-GAGGGTGGCGGCTCT-3'), 400 µM dNTP and 1.25U *Taq* polymerase (5 Prime). Amplification conditions corresponded to: 95°C 5 min, 45 cycles of 95°C 1 min, 36°C 1 min, 72°C 4 min. Generated genetic profiles were analyzed using the Bionumerics software (Applied Maths), in order to group the genetic profiles according to their similarity. A quality control strain was used in each M-13 PCR experiment in order to validate the reputability of the obtained results.

1.3.b Flora monitoring by TTGE

Already available PCR-TTGE protocols were used to follow dynamic of populations (PCR-TTGE) and metabolic activities (RT-PCR-TTGE) of major flora in cheese is described in Parayre et al. (2007). Shortly, highly variable V3 region of 16S ribosomal sequence was targeted by PCR after enzymatic cheese nucleic acid extraction. DNA and cDNA amplicons obtained were separated after gel migration using denaturing temperature conditions for low DNA GC% content. PCR-TTGE and PCR-RT-TTGE patterns were analyzed with BioNumerics software for pattern generation and presumptive identification after comparison with already existing cheese bacterial database.

1.4 Sensory evaluation

Organoleptic qualities of the « control » and « assay » cheeses were evaluated by a sensory expert panel at D+35 (optimal date of consumption) and D+55 (Best Before Date- BBD). The expert panel consisted of 14 people (8 men and 6 women, 40 to 60 years old). Sensorial profiling of the Pont-L'Évêque cheeses was performed according to the NF ISO 13299 norm.

12 categories were evaluated :

Production date		Type	Day of sampling	Abréviation
02/06/2009	A	Control	D36	CA J36
03/06/2009 morning	B		D35	CB J35
03/06/2009 afternoon	C			CC J35
02/06/2009	A	Assay	D36	AA J36
03/06/2009 morning	B		D35	AB J35
03/06/2009 afternoon	C			AC J35
02/06/2009	A	Control	D56	CA J56
03/06/2009 morning	B		D55	CB J55
03/06/2009 afternoon	C			CC J55
02/06/2009	A	Assay	D56	AA J56
03/06/2009 morning	B		D55	AB J55
03/06/2009 afternoon	C			AC J55

2 Results

2.1 Microbiological results

Monitoring was performed in order to observe different microbiological aspects : the behaviour of the traditional starters, the implantation of the strains from the anti-*Listeria* consortium and the incidence of this consortium on the growth of artificially inoculated *Listeria monocytogenes*.

- *Leuconostocs*

Dextran⁺ leuconostocs were only observed in the « assay » samples inoculated with the INRA consortium. The initial for a count was about 10⁶ CFU/g (6 log unit) and stayed constant during the whole process (figure 9).

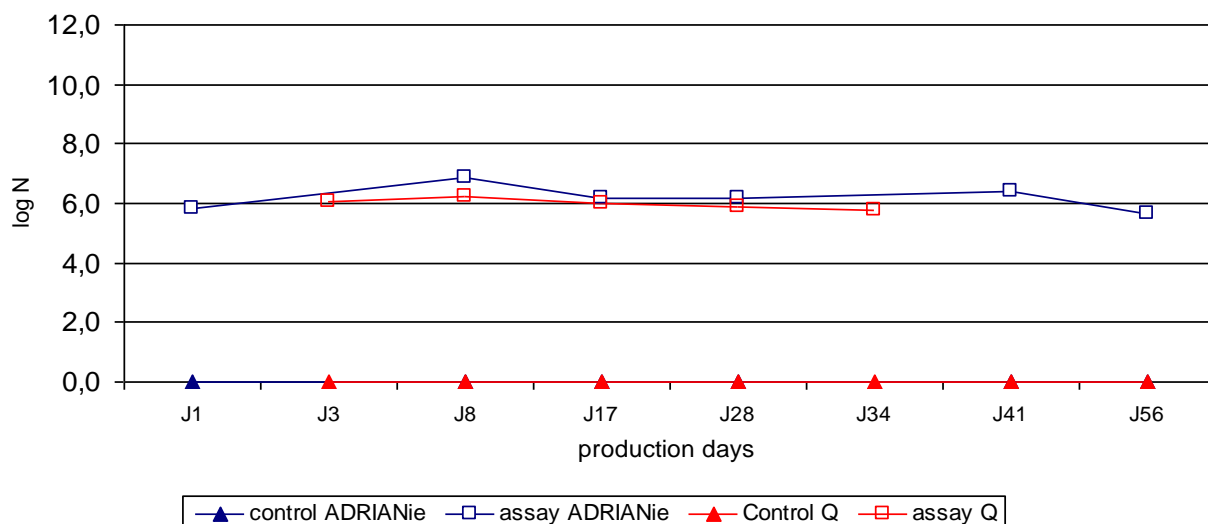


Figure 9 : *Leuconostoc* (dextran⁺) on MSE medium (average of 3 repetitions) during Pont L'Evêque cheesemaking.

Figure 9 illustrates the good implantation of a part of the lactic acid flora of the consortium in the Pont-l'Evêque cheese (*Leuconostoc pseudomesenteroides* and/or *Leuconostoc citreum*). Moreover, no difference of flora was observed between the counts obtained at ADRIA Normandie and ADRIA Développement indicating that the transfer of cheese had no impact on this flora.

- *Lactobacilli*

Lactobacilli were only observed in the « assay » samples inoculated with the INRA consortium. This was expected as this flora is part of the anti-*Listeria* consortium and is not classically used for traditional starter inoculation of Pont l'Evêque cheese. The initial for a count was about 10⁶ CFU/g (6 log) and rose by 2 log units at the beginning of the process (D+6) , then stayed constant during the ripening to reach about 10⁸ CFU/g at the Best Before Date (Figure 10).

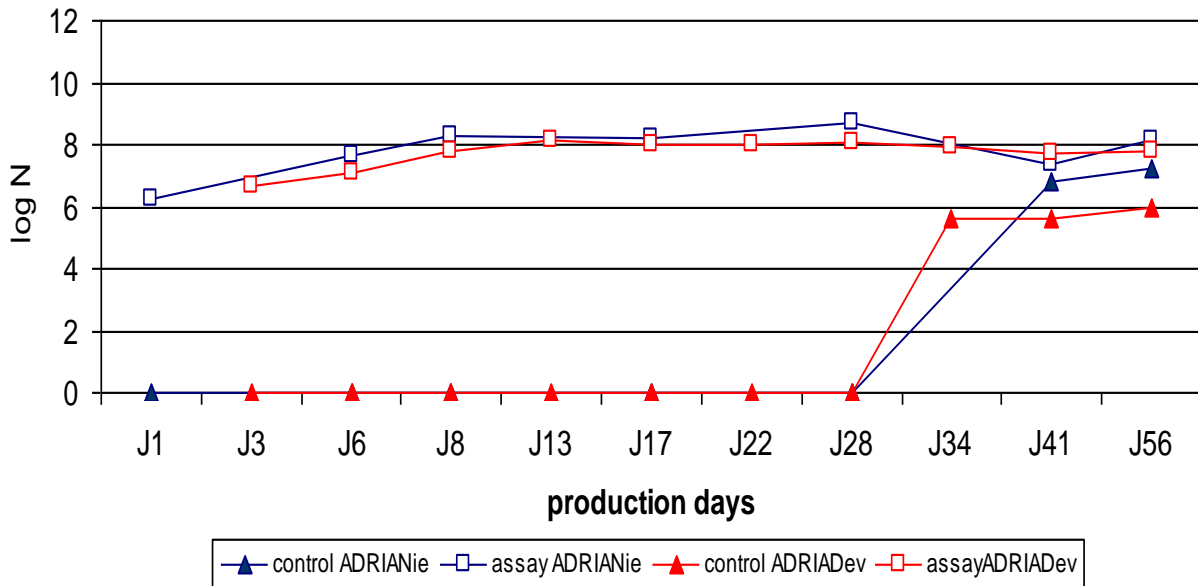


Figure 10 : Lactobacilli on FH medium (average of 3 repetitions) during Pont L'Evêque cheesemaking.

The obtained results indicated the good implantation of the lactobacilli strains of the consortium in the “assay” samples. However, it can be noticed that after 28 days, lactobacilli were observed in the “control” samples. Therefore, it seems that, although the milk was pasteurised, non-starter lactic acid bacteria (NSLAB) corresponding to lactobacilli were present in the milk and able to develop when the cheese environmental conditions were favourable. As observed for leuconostocs, no difference of flora was observed between the counts obtained at ADRIA Normandie and ADRIA Développement.

- **Yeasts and moulds**

Some yeast and mould species were inoculated as traditional starters in the milk during the Pont-l'Evêque cheesemaking process ; they corresponded to *Debaryomyces hansenii* for yeasts and *Geotrichum candidum* for moulds. The initial yeast count was about $4.5 \cdot 10^4$ CFU/g (4,65 log) while the mould initial count was lower than 200 CFU/g (<2,3 log). Both type of flora showed a strong growth until the smearing step (D+6) with average growth rate higher than 4 logs. Then, both floras were stable up to the Best Before Date. At this date, the counts were about $6.5 \cdot 10^8$ CFU/g and $5 \cdot 10^6$ CFU/g, for the yeast and mould flora, respectively (Figures 11et 12).

The obtained results showed similar behaviour for the « control » and « assay » samples for both yeasts and moulds. Therefore, the presence of the anti-*Listeria* consortium did not seem to modify the behaviour of these technological flora in Pont-l'Evêque.

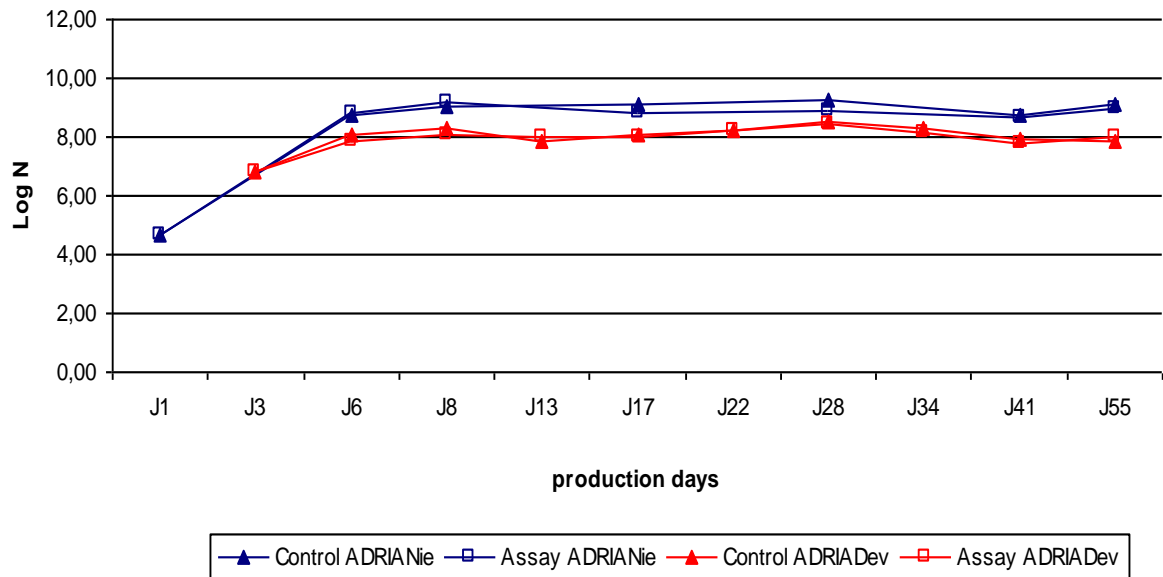


Figure 11 : Yeasts on OGA medium (average of 3 repetitions) during Pont L’Evêque cheesemaking.

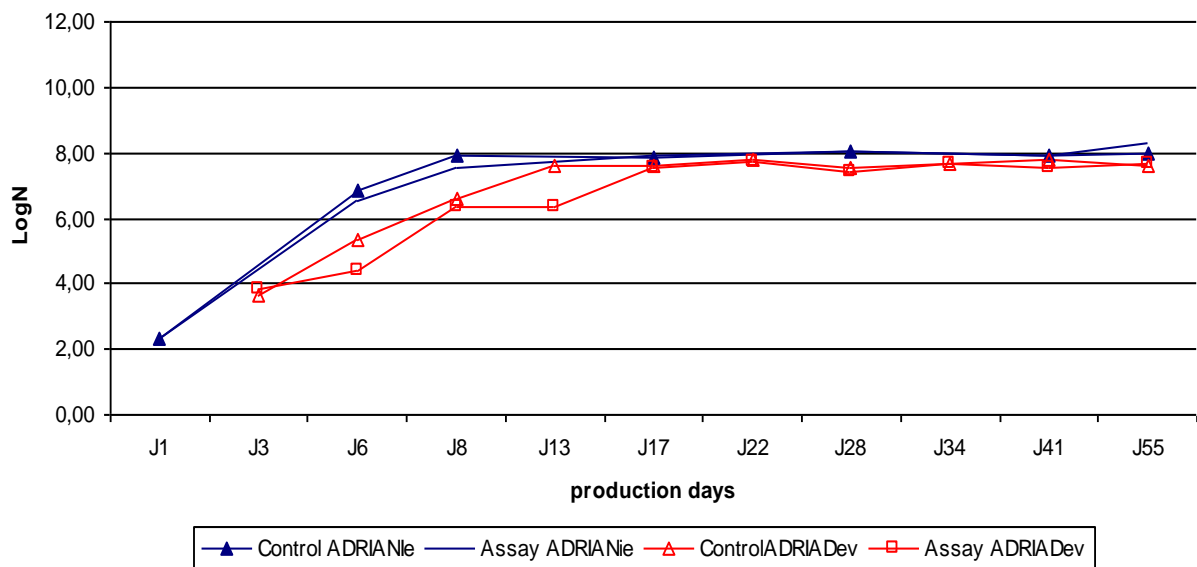


Figure 12 : Moulds on OGA medium (average of 3 repetitions) during Pont L’Evêque cheesemaking.

- *Surface flora*

Surface microorganisms are traditionally used in Pont-l’Evêque cheesemaking technology and especially involved during the ripening phase. They were enumerated during the whole process. The starter cocktail was brought, on one hand, directly in the milk (with addition of the INRA consortium in the assays) and, on the other hand, during the smearing of the “control” and “assays” samples.

The obtained results (Figure 13) showed similar behaviour for the surface flora in presence or not of the INRA consortium. However, it has to be noticed, that the initial count numerated on the « assay » samples was slightly higher ($2.5 \cdot 10^5$ CFU/g) than the “control”

samples (5.10^4 CFU/g) corresponding to a difference of + 0,7 log. This can be explained by the addition of the surface flora included in the anti-*Listeria* consortium in the “assays” conditions. After the smearing step (D+6), both curves are identical.

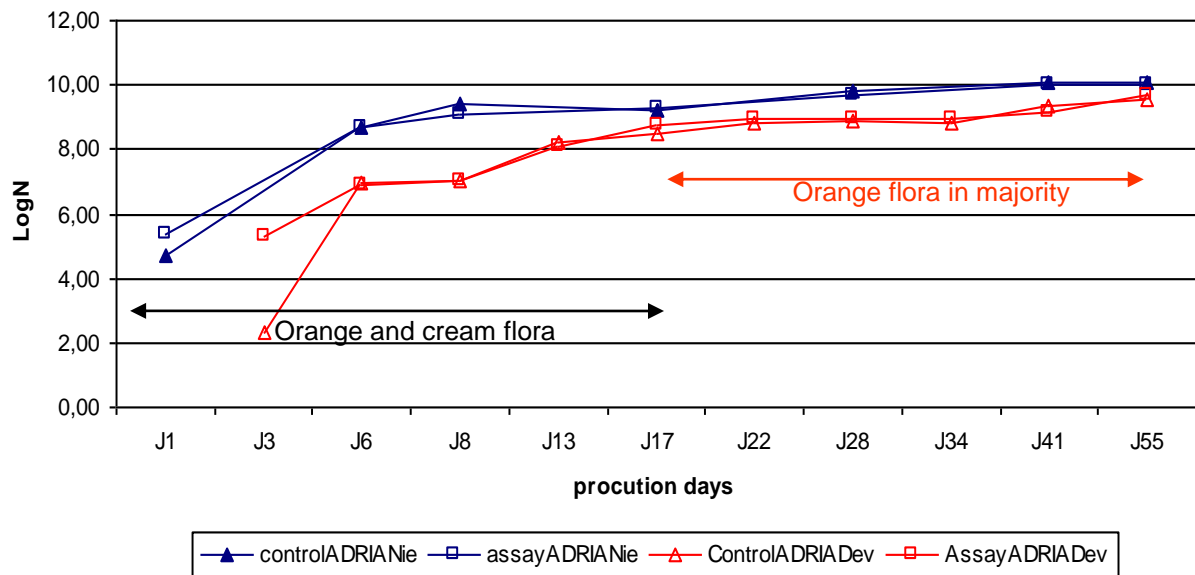


Figure 13 : Surface flora on CRBM medium (average of 3 repetitions) during Pont L’Evêque cheesemaking.

In all modalities (“control” and “assay” samples), we observed the presence of an orange flora all along the cheesemaking process, while a cream flora was only observed the first 17 days of the process. The INRA consortium did not seem to have an influence on the development of the technological surface flora.

- ***Gram-negative flora***

Concerning Gram-negative flora, the data obtained (Figure 14) indicated that the growth curve obtained for the “controls” were similar to those of the “assays”. Thus, the INRA consortium did not seem to have an influence on the development of Gram-negative flora, a non starter flora associated with cheese production. An apparent slower bacterial growth on ADRIA Développement site until day 17 was observed; however, similar population levels were obtained after 55 days of cheese conservation.

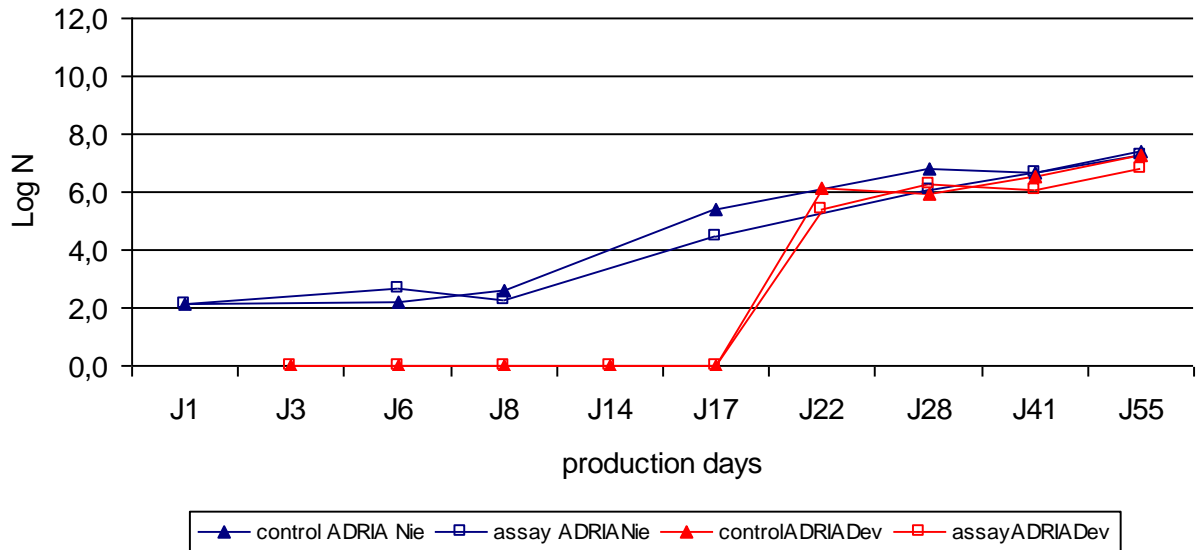


Figure 14 : Gram-negative flora on PCAi medium (average of 3 repetitions) during Pont L'Evêque cheesemaking.

- *Listeria monocytogenes*

During this study, the *L. monocytogenes* strain (Saint Nectaire cheese origin) artificially inoculated was monitored during the Pont-l'Evêque process. *L. monocytogenes* counts of 3 production batch are reported in table 13 for cheese "control" and "assay".

Table 13: *L. monocytogenes* counts for cheese control (without INRA consortium) and cheese assay (without INRA consortium) are reported as mean and standard deviations for the three production batches

time (days)	<i>L. monocytogenes</i> counts (logCFU/g)			
	mean control	+/-	mean assay	+/-
0	2,67E+01	2,08E+01	1,67E+01	1,15E+01
3	2,50E+01	7,07E+00	/	/
6	1,00E+01	/	1,20E+01	/
11	1,37E+03	9,83E+02	8,65E+02	1,18E+03
14	1,58E+04	1,18E+04	5,26E+03	4,30E+03
20	1,18E+05	7,83E+04	3,97E+04	2,89E+04
25	2,67E+05	1,60E+05	3,83E+04	2,39E+04
31	6,20E+05	3,90E+05	1,23E+05	9,07E+04
38	1,77E+06	3,21E+05	6,83E+04	8,09E+04
53	1,97E+06	1,78E+06	1,01E+06	1,47E+06

No significative difference is observed between *L. monocytogenes* counts at the beginning and at the end of ripening. Nevertheless at D+25, D+31 and D+38 it is worth noting that a difference was observed between both conditions, this difference in bacterial counts reaches up to 1.5 logs at D+38 with levels of $6.8 \cdot 10^4$ CFU/g and $1.7 \cdot 10^6$ CFU/g for the "control" and "assay" samples, respectively. However taking into consideration the low level of *L. monocytogenes* inoculation (5 CFU/cm^2), the variability in lag time and sampling, only little differences were observed between « control » and « assay » samples as shown in Figure 15.

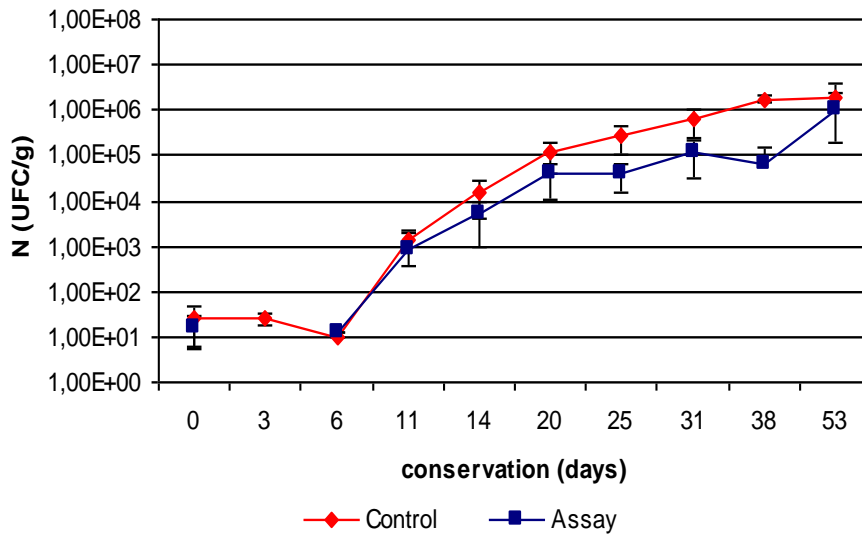


Figure 15 : *Listeria monocytogenes* on ALOA medium (average of 3 repetitions) during Pont L’Evêque cheesemaking.

2.2 Physico-chemical results

- *pH*

At the beginning of the cheesemaking process, the pH corresponded to 5, this value was constant until D+28, then it raised of 1 unit at the end of the process to reach pH6 at D+55 (Figure 16). No difference was observed between the “control” and “assay” samples.

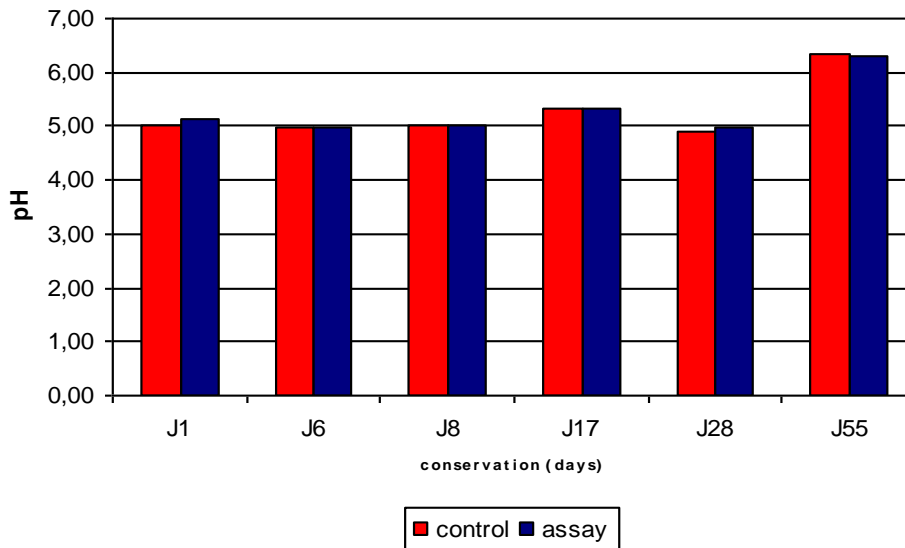


Figure 16 : pH monitoring during Pont L’Evêque cheesemaking..

- A_w

The average water activity of the “control” and “assay” samples was about 0,980. Significant variations were observed during the production process for each condition (“controls” and “assays”); however, the INRA consortium did not seem to have an influence on the tested physico-chemical parameters (Figure 17).

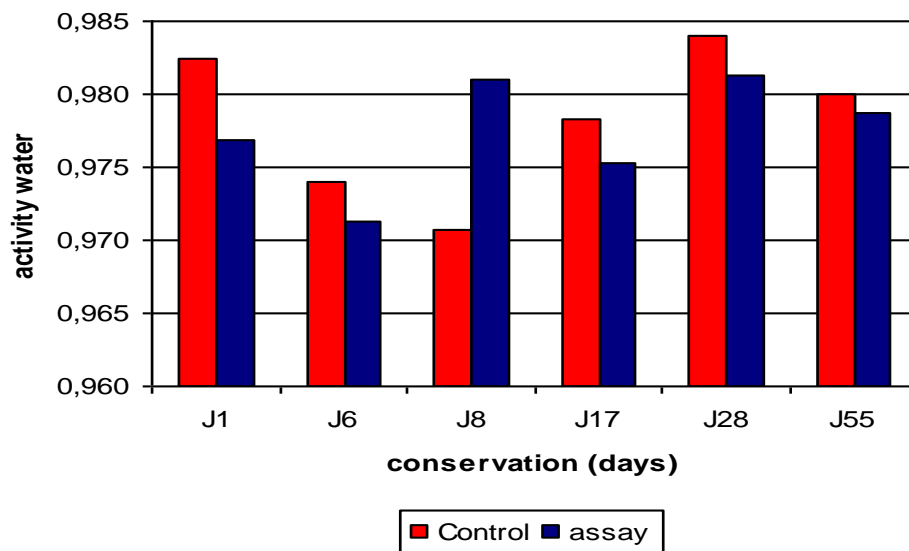


Figure 17 : A_w monitoring during Pont L’Evêque cheesemaking..

2.3 Flora monitoring by molecular biology

1.3.a Consortium monitoring by M13-PCR

A total of 100 isolates, including the 14 consortium strains as well as 86 strains isolated during the Pont-L’évêque production, were analyzed by M13-PCR. The generated genetic profiles were then integrated in the bioinformatic software Bionumerics to perform a grouping analysis. In a first step, the genetic profiles of the consortium strain were analyzed. A good genetic profile differentiation was observed for all strains of the consortium with the exception of *S. equorum* RPF6 / *A. nicotianae* TU9 / *B. rhamnosum* PCA7 and *L. plantarum* FH3 / *L. farciminis* FH4 (Figure 18). Indeed, the similarity observed between the genetic profiles obtained for pour *S. equorum* RPF6 / *A. nicotianae* TU9 / *B. rhamnosum* PCA7 (PM13-3 group) was >90% as the one observed for *L. plantarum* FH3 / *L. farciminis* FH4 (PM13-8 group). Therefore, the implantation of these strains was not followed individually but at the group level.

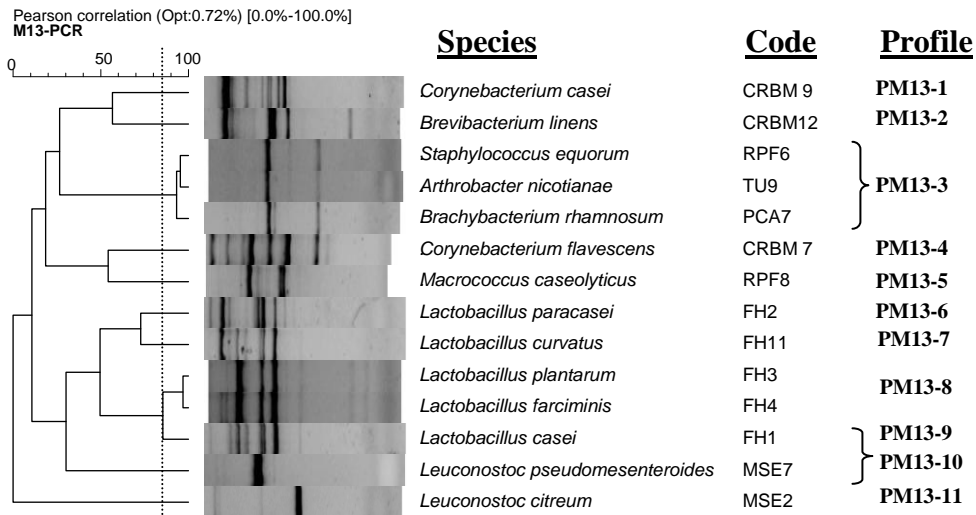


Figure 18. M13-PCR genetic profiles obtained for the 14 consortium strains. The dendrogram was constructed using the Pearson correlation and UPGMA, a threshold value of 85% was applied.

In a second time, 86 strains isolated, between D+14 and D+55, from Pont-L'évêque « assay » cheeses were characterized by M13-PCR. The obtained genetic profiles were integrated in Bionumerics and compared to those of the consortium strains.

- ***Lactobacilli***

Firstly, the implantation of the consortium strains belonging to the 5 species of the *Lactobacillus* genus was studied (Figure 19). The *L. curvatus* strain FH11 (profile PM13-7) was observed at the beginning of the process and was constantly found until D+28 ; at the end of the ripening (D+40 and D+55), this strain was not detected. Concerning the *L. plantarum* FH3 / *L. farciminis* FH4 strains (same M1-PCR profile, PM13-8 group), the implantation was apparently weak at the beginning of the production (only 1 to 2 strains observed at D+1, D+17 and D+28) then, higher at D+40. For *L. paracasei* FH2, this strain was only observed at the end of the cheesemaking process (only D+55). *L. casei* FH1 was not detected among the tested isolates. Finally, the implantation of the consortium strains belonging to the *Lactobacillus* genus was good and represented ~42% of the tested isolates.

- ***Leuconostocs***

Concerning *Ln. citreum* MSE2 and *Ln. pseudomesenteroides* MSE7 (Figure 18), only 3 strains grouped with *Ln. pseudomesenteroides* MSE7 according to their genetic profile. This strain was only observed at the end of the cheesemaking (D+40 and D+55). No strain presenting a genetic profile corresponding to *Ln. citreum* was detected among the 86 tested strains. These results have to be put in perspective according to the MSE counts results indicating a good implantation of dextran⁺ leuconostocs.

- ***Surface flora***

Concerning surface flora, the implantation of the *Corynebacterium casei* CRBM9, *C. flavescens* CRBM7, *Brevibacterium linens* CRBM12, *Arthrobacter nicotianae* TU9, *Brachybacterium rhamnosum* PCA7, *Staphylococcus equorum* RPF6 and *Macrococcus caseolyticus* RPF8 strains was monitored (Figure ?). Only 2 strains, *B. linens* CRBM12 and *C.*

flavescens CRBM7, were observed. *C. flavescens* CRBM7 was present at the beginning of the cheesemaking process (D+1 and D+8); while, *B. linens* was present during (from D+17 to D+40) and especially at the end (D+55) of the ripening.

A global analysis of the obtained results indicated that, 65% of the isolates analyzed by M13-PCR corresponded to consortium strains. *Lactobacillus* strains presented the best implantation in the « assay » cheese (~42% of tested strains), followed by surface flora (~20%, but only *B. linens* and *C. flavescens*) and *Leuconostoc* strains (~3.5%, only *L. pseudomesenteroides*).

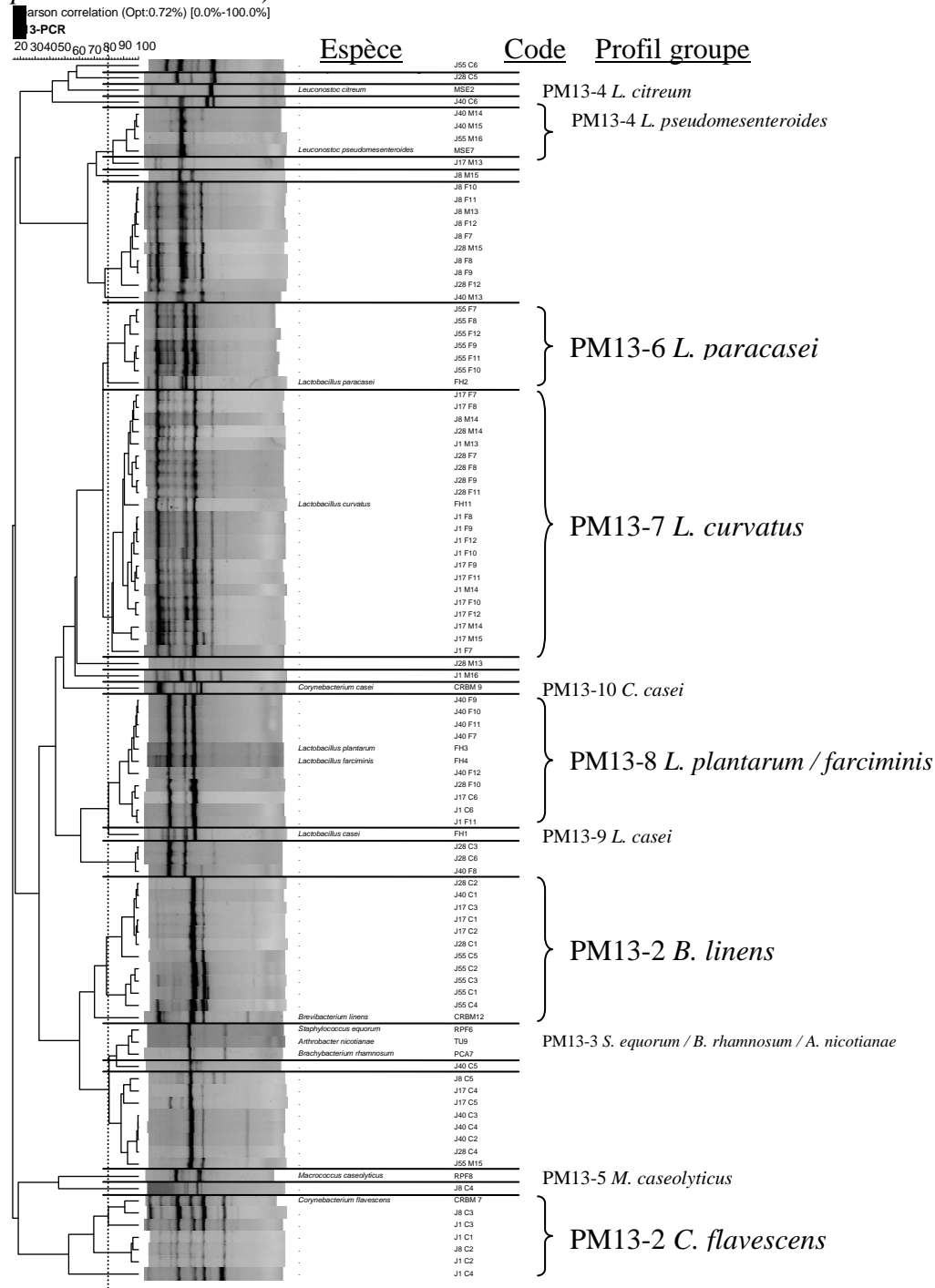


Figure 19. Genetic profiles generated by M13-PCR for the consortium strains and the cheese isolates. The dendrogram was constructed using the Pearson correlation and UPGMA, a threshold value of 80% was applied.

1.3.b Flora monitoring by TTGE

The use of PCR-TTGE enables the study of major population in cheese ecosystems. After migration of the amplicons in a denaturing gel, profiles are computerised and analysed by BioNumerics software to enable comparison of TTGE bands, i.e. comparison of population present. Figure 20 represents the profile of migration bands obtained for the INRA consortium as well as the band which has been considered in red when more than one band was observed for a single strain. Eventhough several purifications have been done, multiple bands obtained for some strain is associated to multiple copies of ribosomal operon. Note that considering a single band per strain, marked in red, did not enable distinction between *Lactobacillus* FH3-FH4, *Lactobacillus* FH2-FH11, *Lactobacillus* FH1-*Arthrobacter* Tu9 and *Leuconostoc* MSE7-*Staphylococcus* RPF6 in tested conditions. Similarly, due to close band migration distance, no distinction was possible between *Listeria monocytogenes* SN167 and *Leuconostoc citreum* MSE2 (figure 21)

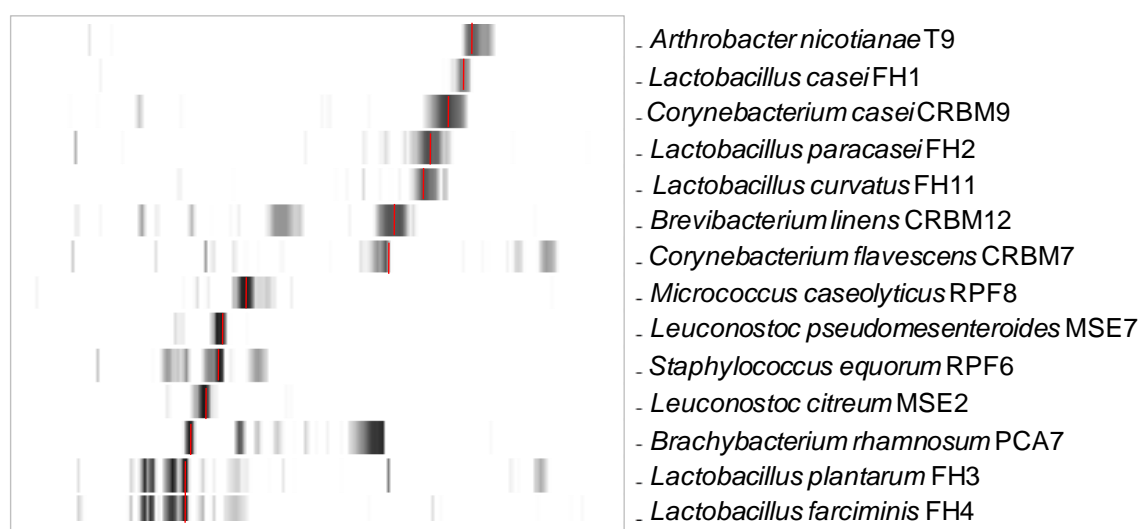


Figure 20: migrating TTGE bands obtained for each strain of INRA “anti-*Listeria*” consortium after analysis with BioNumerics software. Red bands indicate migration distance considered for each strain

Note that the molecular approach used only gives information related to the dynamic of major populations as band intensity is proportional to population size. Considering bacterial counts obtained along cheese ripening, *L. monocytogenes* counts are low as compared other flora. *Listeria* population reaches 6 log at the end of shelf life while lactobacilli, yeast-moulds and surface flora reach, respectively 8, 9 and 10 log. The objective of PCR-TTGE migration profiles being to illustrate dynamics of major population present (PCR-TTGE) and associated metabolic activities (RT-PCR-TTGE).

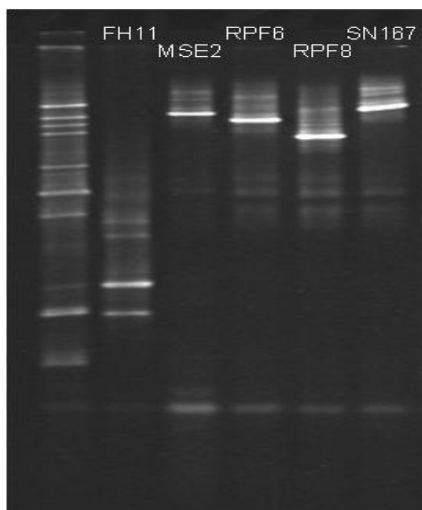


Figure 21: TTGE gel profile obtained after migration of strain FH11, MSE2, RPF6, RPF8 and *L. monocytogenes* SN167 in tested conditions

Profiles reported in figure 22 highlight population dynamic and implantation for surface and core of “control” and “assay”cheeses at day D0, D+3, D+6, D+11, D+14, D+20, D+25, D+31, D+38 and D+53. Population switches are observed as for instance in core sample at D+20. At first sight more diversity is observed in control cheeses. In order to better follow the dynamic of population and associated activities, computer analysis provided by BioNumerix software is presented as 0-1 matrices for global cheese ecosystem (figure 23) and INRA consortium (figure 24). While no differences could be observed between assay and control cheese based on bacterial counts, TTGE analysis show different population dynamics and activity when INRA consortium is inoculated or not. Even though not exhaustive, TTGE database allows presumptive identification of most bands. Non-identified bands have been mentioned as NA in figure 23. Because TTGE does not allow distinction between closely related strains (*Lb farciminis* and *Lb plantarum*) or strains for which targeted ribosomic sequences co-migrate (*Leuconostoc pseudomesenteroides* and *Staphylococcus equorum*), as well as lactic bacterial strains used in Pont l’Evêque cheese manufacture (annex 1), ecosystem analysis remains complex. Nevertheless it could be noted a low implantation of INRA consortium with few active strains in assay cheeses for both core and surface. Comparing profiles, control cheese ecosystem seems more complex than assay cheese ecosystem yielding to an optimal development of Pont l’Evêque flora in the absence of INRA consortium. Surprisingly the core ecosystem switch in assay cheese seems to correspond at day 20, to the disappearance of bands associated to the INRA consortium strains and appearance of NA bands, phenomenon which is not observed with control cheese suggesting an impact of INRA consortium in studied cheese ecosystems.

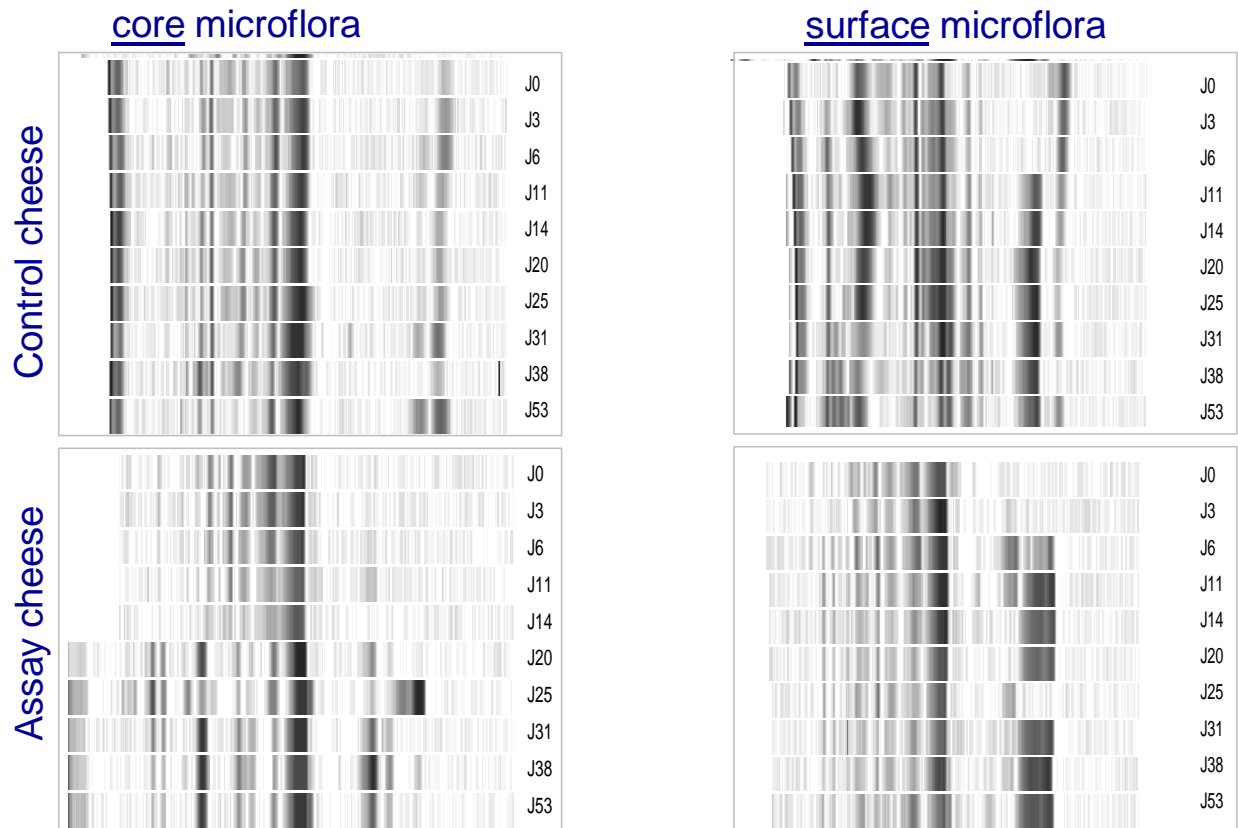


Figure 22: PCR-TTGE profiles obtained after computer analysis regarding sampling of cheese surface and core of “control” and “assay” of Pont l’évêque along shelf-life, i.e. day D0, D+3, D+6, D+11, D+14, D+20, D+25, D+31, D+38 and D+53.

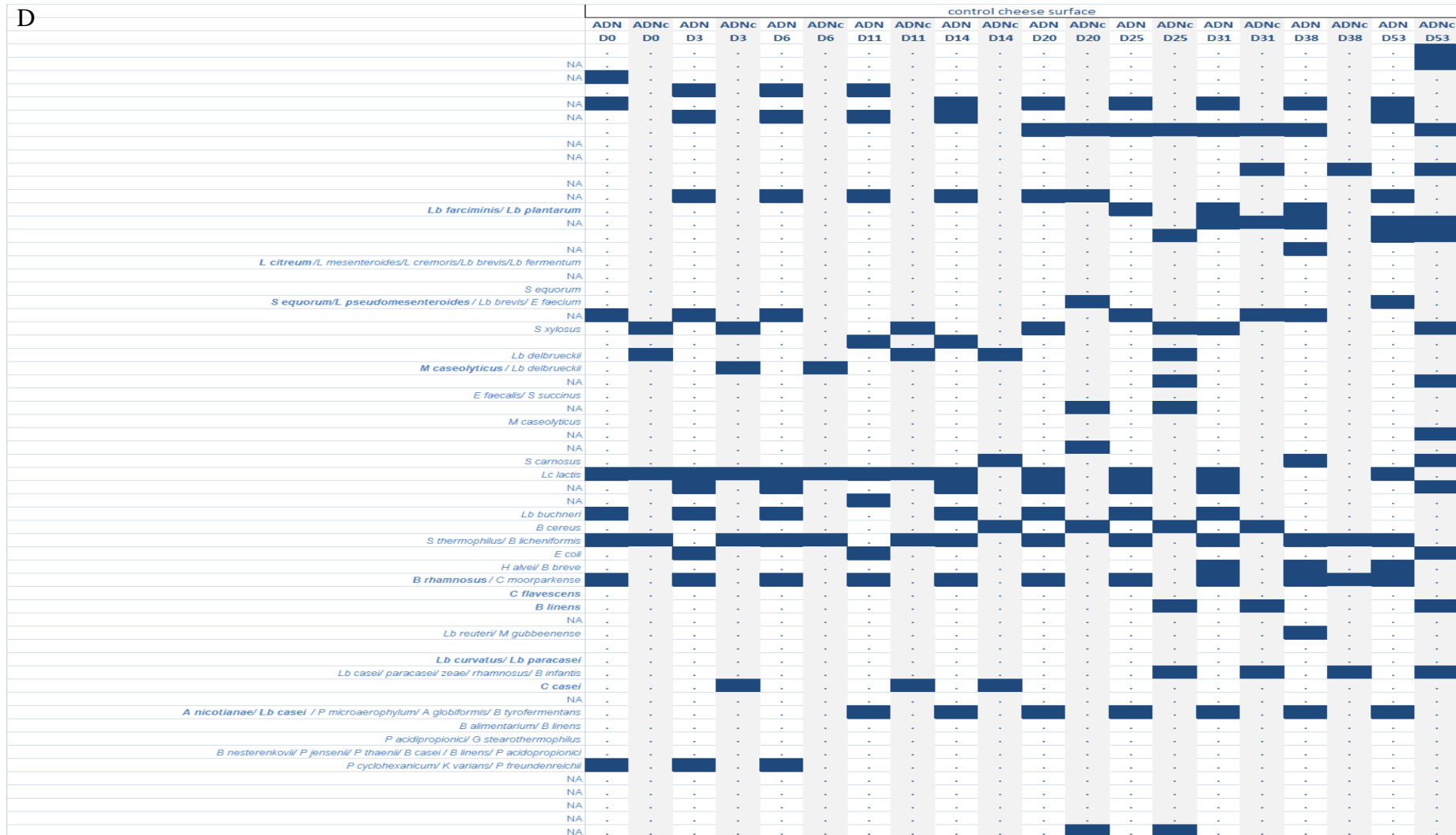


Figure 23: PCR-TTGE and RT-PCR-TTGE(ADN) BioNumerix profile analysis presented as a 0-1 matrix to study respectively population dynamics and metabolic activity of surface and core cheese ecosystem. Analysis is presented for cheese core ecosystem for assay (A) and control (B) as well as surface ecosystem for assay (C) and control (D) along shelf-life. INRA consortium strains have been indicated in bold while bands for which no identification has been attributed are indicated as NA.

cheese	"anti listeria" consortium	ADN										ADNc									
		sampling (day)										sampling (day)									
		D0	D3	D6	D11	D14	D20	D25	D31	D38	D53	D0	D3	D6	D11	D14	D20	D25	D31	D38	D53
Assay Core	FH3 Lactobacillus plantarum / FH4 Lactobacillus farciminis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MSE2 Leuconostoc citreum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MSE7 Leuconostoc pseudomesenteroides/ RPF6 Staphylococcus equorum	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	RPF8 Micrococcus caseolyticus	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PCA7 Brachybacterium rhamnosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM7 Corynebacterium flavescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM12 Brevibacterium linens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	FH2 Lactobacillus paracasei /FH11 Lactobacillus curvatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM9 Corynebacterium casei	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	FH1 Lactobacillus casei /TU9 Arthrobacter nicotianae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control Core	FH3 Lactobacillus plantarum / FH4 Lactobacillus farciminis	1	0	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0
	MSE2 Leuconostoc citreum	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0
	MSE7 Leuconostoc pseudomesenteroides/ RPF6 Staphylococcus equorum	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	RPF8 Micrococcus caseolyticus	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0
	PCA7 Brachybacterium rhamnosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM7 Corynebacterium flavescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM12 Brevibacterium linens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	FH2 Lactobacillus paracasei /FH11 Lactobacillus curvatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM9 Corynebacterium casei	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	FH1 Lactobacillus casei /TU9 Arthrobacter nicotianae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Assay Surface	FH3 Lactobacillus plantarum / FH4 Lactobacillus farciminis	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1
	MSE2 Leuconostoc citreum	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	0	1	0
	MSE7 Leuconostoc pseudomesenteroides/ RPF6 Staphylococcus equorum	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1
	RPF8 Micrococcus caseolyticus	1	1	0	0	1	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0
	PCA7 Brachybacterium rhamnosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM7 Corynebacterium flavescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM12 Brevibacterium linens	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	FH2 Lactobacillus paracasei /FH11 Lactobacillus curvatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM9 Corynebacterium casei	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	FH1 Lactobacillus casei /TU9 Arthrobacter nicotianae	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	1	1	0	0
Control Surface	FH3 Lactobacillus plantarum / FH4 Lactobacillus farciminis	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
	MSE2 Leuconostoc citreum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MSE7 Leuconostoc pseudomesenteroides/ RPF6 Staphylococcus equorum	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
	RPF8 Micrococcus caseolyticus	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	PCA7 Brachybacterium rhamnosum	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
	CRBM7 Corynebacterium flavescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM12 Brevibacterium linens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
	FH2 Lactobacillus paracasei /FH11 Lactobacillus curvatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CRBM9 Corynebacterium casei	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
	FH1 Lactobacillus casei /TU9 Arthrobacter nicotianae	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0

Figure 24: Implantation of INRA consortium strains is presented as a 0-1 matrix provided after BioNumerix analysis for core and surface sampling in “control” and “assay” pont l’Evêque cheeses. PCR-TTGE(ADN) and RT-PCR-TTGE(ADNc) enable the study of populations as well as associated metabolic activity along shelf-life

2.4 Sensory evaluation

2.4.a Sensorial characteristics at optimal date of consumption (D+35 and D+36)

○ Study of the 6 samples

A 2 factor (subject and product) variance analysis and a Newman-Keuls test were performed in order to identify the descriptors allowing to differentiate the tested cheeses and to obtain a ranking of the cheese based on the average values obtained.

The ANOVA analysis revealed significant differences for 6 descriptors (following table) :

	Descriptor	Newman-Keuls (5%) (1) →
	Red smear quantity	CB CC <u>CA</u> AC AB <u>AA</u>
SURFACE ASPECT	Red smear homogeneity	CC <u>AB</u> CB AC <u>CA</u> <u>AA</u>
	Colour intensity	<u>CB</u> CC AB CA AC <u>AA</u>
	Geotrichum quantity	<u>AA</u> <u>CB</u> AB AC CC CA
SECTION ASPECT	Ripening level	AC CC AB <u>CB</u> <u>AA</u> <u>CA</u>
SAVOUR	Saltiness	<u>AA</u> <u>AA</u> <u>AB</u> <u>AB</u> <u>AC</u> <u>AC</u>

(1) The test is given with an error risk of 5%.

Conditions underlined did not show significant differences.

The main observed concerned the cheese aspect ; while, no difference was observed for odour, firmness and aromas. Significant different criteria were as follows :

- Red cheese smear quantity:

CB showed significantly less red smear starter than CA ; no difference was observed between the 3 “assay” samples. AA showed significantly more red smear starter than CB and CC.

- Red smear homogeneity:

For this descriptor, differences were only observed for CC and AA, AA being more homogenous than CC. No differences between “control” and “assay” samples was observed.

- Colour intensity:

Only AA was significantly darker than the rest of the samples..

- Geotrichum quantity:

CA and CC showed significantly higher presence of *Geotrichum* than AA.

- Ripening level:

Only CA and AC were significantly different, CA was more ripened than AC.

- Saltiness:

Concerning the salty sensation, CC was significantly more salty than AA and CA.

- *Study of the control as whole C (CA, CB, CC combined) and the assay A as a whole (AA, AB, AC combined).*

	Descriptor	Newman-Keuls (5%) (1) →
	Red smear quantity	<u>C</u> <u>A</u>
SURFACE ASPECT	Colour intensity	<u>C</u> <u>A</u>
	Yeast quantity	<u>A</u> <u>C</u>
SECTION ASPECT	Core colour	<u>C</u> <u>A</u>

(1) The test is given with an error risk of 5%.
Conditions underlined did not show significant differences.

The “control” and “assay” samples, only differed by aspect criteria. Assays showed significantly more red smear starter and a more intense colour of the core and surface. Controls showed a higher presence of yeast on the surface. Finally, no difference between the “control” and “assay” samples was observed for odour, taste and texture.

- *Conclusion at D+35 and D+36*

At D+35 and D+36, only aspect differences were observed between the “control” and “assay” samples, no difference was observed for odour, taste and texture.

The period of production seems to have an incidence on the sensory characters of the cheese. Indeed, significant differences were observed between repeats :

Repeat 1 = R1 = CA-AA, J 36
Repeat 2 = R2 = CB-AB, J 35 morning
Repeat 3 = R3 = CC-AC, J 35 afternoon

	Descriptor	Newman-Keuls (5%) (1) →
	Red smear quantity	<u>R2</u> <u>R3</u> <u>R1</u>
	Red smear homogeneity	<u>R2</u> <u>R3</u> <u>R1</u>
SURFACE ASPECT	Colour intensity	<u>R2</u> <u>R3</u> <u>R1</u>
	Flat	<u>R1</u> <u>R2</u> <u>R3</u>
SECTION ASPECT	Ripening level	<u>R3</u> <u>R2</u> <u>R1</u>
TEXTURE	Smoothness	<u>R2</u> <u>R3</u> <u>R1</u>
FLAVOUR	Saltiness	<u>R1</u> <u>R2</u> <u>R3</u>

(1) The test is given with an error risk of 5%.
Conditions underlined did not show significant differences.

The cheese produced first (R1, 02/06/2009, sensory evaluation at D+36) showed more red smear starter, a deeper colour and a higher ripening level. The cheese produced last (R3, 03/06/2009 afternoon) were flatter and saltier than those produced on the 02/06/2009 and even

from those produced the same day but in the morning (different milk). Thus, at D+35 and D+36, the cheese were visually distinct according to their production period.

2.4.b Sensorial characteristics at Best Before Date (D+55 and D+56)

○ Study of the 6 samples

	Descriptor	Newman-Keuls (5%) (1) →
	Red smear quantity	<u>CB</u> <u>AA</u> <u>AC</u> <u>CA</u> <u>CC</u> <u>AB</u>
	Colour intensity	<u>CB</u> <u>AA</u> <u>CC</u> <u>AC</u> <u>AB</u> <u>CA</u>
SURFACE ASPECT	Geotrichum quantity	<u>AC</u> <u>AB</u> <u>CB</u> <u>CC</u> <u>CA</u> <u>AA</u>
	Yeast quantity	<u>CB</u> <u>AB</u> <u>AC</u> <u>CA</u> <u>CC</u> <u>AA</u>
	Flat	<u>CA</u> <u>AA</u> <u>AC</u> <u>CC</u> <u>CB</u> <u>AB</u>
SECTION ASPECT	Runny	<u>AB</u> <u>CB</u> <u>AA</u> <u>CA</u> <u>AC</u> <u>CC</u>
SIDE AROMA	Stable, hay	<u>AC</u> <u>CB</u> <u>AB</u> <u>CC</u> <u>AA</u> <u>CA</u>
TEXTURE	Firmness	<u>AC</u> <u>CC</u> <u>CA</u> <u>CB</u> <u>AA</u> <u>AB</u>

(1) The test is given with an error risk of 5%.
Conditions underlined did not show significant differences.

For all products, no significant difference was observed concerning the flavour and taste. The only significant differences indicated that:

- Control A presented a slightly higher stable aroma.
- Control B was firmer and showed more red smear starter.
- Control C exhibited more red smear starter and yeast. It was considered more runny.
- Assay A presented a higher quantity of *Geotrichum* and yeast and stronger stable odour.
- Assay B presented a flat aspect.
- Assay C was quite runny.

○ Study of the control as whole C (CA, CB, CC combined) and the assay A as a whole (AA, AB, AC combined).

	Descriptor	Newman-Keuls (5%) (1) →
SECTION ASPECT	Runny	<u>A</u> <u>C</u>
AROMAS	Stable, hay	<u>A</u> <u>C</u>

(1) The test is given with a error risk of 5%.

Conditions underlined did not show significant differences.

When all “control “ samples are combined (C) and the “ assay “ samples too (A), only 2 sensory were differentiating them. The “control” was more runny and presented stronger stable aroma than the “assay”. Actually, a variability was observed among the 3 “control” samples and among the 3 “assay” samples.

○ **Conclusion at D+55 and D+56**

As observed at D+35/36, the recorded differences related mainly to the cheese aspect, but some differences in texture (runny or firm) were also established. However, the differences entre between the « control » and the assay » samples are not clear. Indeed, differences were observed between the repeats of either the « control » or the « assay ».

Thus, by studying the repeats (CA-AA, D+56) / (CB-AB, D+55 morning) / (AC-AC, D+55 afternoon), some significant differences were observed:

Repeat 1 = R1 = CA-AA, D+56

Repeat 2 = R2 = CB-AB, D+55 morning

Repeat 3 = R3 = CC-AC, D+55 afternoon

	Descriptor	Newman-Keuls (5%) (1) →
SURFACE ASPECT	Red smear quantity	<u>R2</u> <u>R1</u> <u>R3</u>
	<i>Geotrichum</i> quantity	<u>R3</u> <u>R2</u> <u>R1</u>
	Yeast quantity	<u>R2</u> <u>R3</u> <u>R1</u>
SECTION ASPECT	Flat	<u>R1</u> <u>R3</u> <u>R2</u>
	Runny	<u>R2</u> <u>R1</u> <u>R3</u>
SIDE AROMA	Global intensity	<u>R3</u> <u>R2</u> <u>R1</u>
	Stable, hay	<u>R2</u> <u>R3</u> <u>R1</u>
TEXTURE	Firmness	<u>R3</u> <u>R1</u> <u>R2</u>

(1) The test is given with an error risk of 5%.

Conditions underlined did not show significant differences.

Except for the quantity of *Geotrichum*, the global and stable aroma intensity (R1), no clear difference was observed according to the production period.

At D+55/56, the anti-*Listeria* consortium did not seem to have an incidence on the smell and taste of the inoculated cheese (“Assay“); the only observed differences corresponded to the cheese aspect. Moreover, it is important to note that the variability observed among the repeats of “control” samples and among the repeats of the “assay” samples was higher than the one observed between the control and assay samples.

3 Conclusion

The microbiological and physico-chemical analyses did not show significant differences between the “control” and “assay” samples. Therefore, the anti-*Listeria* consortium selected by INRA did not modify the development of the Pont-l’Evêque technological flora.

While no significant difference could be observed with microbial counts and physico-chemical analysis, molecular based methods highlight differences in control and assay cheese population dynamics. Even though microbial interactions occurring in cheese ripening are rather difficult to analyse, molecular grouping shows good implantation of the consortium strains which seems controlled by the growth of dextran⁺ leuconostocs and lactobacilli (flora specific to the consortium). Similarly, the presence of INRA consortium yields to different PCR-TTGE and RT-PCR-TTGE profiles especially in core ecosystem where a population switch is observed only in presence of the consortium. This study allows demonstrating the possible implantation in Pont-l’Evêque cheese of a cocktail of strains specifically selected for another cheesemaking technology : Saint-Nectaire. This was especially confirmed for lactobacilli (dominance of *L. curvatus* FH11) by the results of implantation monitoring obtained by M13-PCR.

The sensory evaluation of the cheese did not reveal, except for the cheese aspect, any major differences between the “control” and “assay” samples. The more significant differences were rather correlated to the production date and hence to the milk used (3 different milks were used).

Concerning the *Listeria monocytogenes* inhibitory effect of the selected consortium, at the Best Before Date, no differences were observed between the « controls » and the inoculated cheeses (« assays »). However, it seems that the presence of the consortium could reduce the *L. monocytogenes* growth (lower populations at D+38 in the “assays”). These results are to be appreciated in parallel of those of the implantation monitoring. Indeed, we observed a weak implantation of the surface flora belonging to the anti-*Listeria* consortium that could explain the reduced incidence of the consortium on *L. monocytogenes* observed during this study. This study regards *L. monocytogenes* artificial contamination simulating post contamination on cheese surface, different impact of the consortium could have been observed simulating a milk contamination with *L. monocytogenes*.

General conclusion

The selected consortium had a limited anti-*Listeria* effect on the surface of Saint-Nectaire cheeses that can be explained by a competition between microbial flora and flora from raw milk. The raw milk flora may also have an anti-*Listeria* effect. In Pont l’Evêque cheese, the low inhibition at the end of ripening can be due to a low implantation of the consortium at the surface; moreover, the traditional starter might stimulate *L. monocytogenes* growth by consumption of acids. This study confirmed the difficulties for inhibiting *L. monocytogenes* at the surface of cheeses as the pH favours its growth. The pH was 7.2 in Saint Nectaire cheeses ripened 28 days and the pH was 6 in Pont l’Evêque cheese at 55 days.

In this study the maximum level of *L. monocytogenes* was generally higher than the one classically observed in naturally contaminated cheeses. This is comforted by the assay performed in Cantal cheeses. Indeed, in Cantal cheeses prepared in a farm presenting recurrent cheese contamination by *L. monocytogenes* (low level <10 UFC/g), this bacteria was no more detected

after 3 months in cheeses with consortium while the pathogen was still present in some samples without consortium.

Yet, this first application of the anti-*Listeria* consortium can lead to different perspectives.

In the case of *Listeria* post-contamination: assays with only the anti-*Listeria* consortium (without traditional starters) as well as use of the anti-*Listeria* surface flora (7 strains) for smearing could be performed. As the surface flora were selected for Saint Nectaire, it would be important to evaluate if the obtained cheese would be acceptable Pont l'Evêque.

In the case of milk contamination: the good implantation of lactobacilli in Pont l'Evêque cheeses suggests that the consortium could be of interest for milk contamination by *Listeria* (raw milk) as it was shown in WP2A in experimental on cooked pressed cheeses Saint-Nectaire . The selected anti-*Listeria* consortium can be a preventive tool in raw milk cheese-producing farms confronted with recurrent *L. monocytogenes* contamination of their milk.

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Annex 1

Traditional starters used for the Pont l'Evêque production

In the milk :

- Lactic flora : Flora Danica (Chr Hansen)/ PAL ST (Standa)
 - *Flora Danica (*Lc. lactis* ; *Lc.cremoris* ; *Lc diacetyllactis* ; *Ln. mesenteroides cremoris* (dext-))
 - *PAL ST (thermophilic streptococci)
- Yeast : DH (Cargill) : *Debaryomyces hansenii*
- Geo 13 Lyo Choozit™ (Danisco) : *Geotrichum candidum*
- Surface flora : Choozit™PLA Lyo (Danisco)
 - **Brevibacterium linens* (pigmentation du crème au orange)
 - **Arthrobacter nicotianae* (jaune)
 - **Debaryomyces hansenii*
 - **Geotrichum candidum*

Smearing step :

- Choozit™PLA Lyo (Danisco)