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### **Report describing the preparation of microbial consortium adapted to farm and SME production**

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<b>RE</b>	Restricted to a group specified by the consortium (including the Commission Services)	
<b>CO</b>	Confidential, only for members of the consortium (including the Commission Services)	

## Deliverable\_D6.1.1.1

### Report describing the preparation of microbial consortium adapted to farm and SME production

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#### **Summary**

This deliverable describes the optimisation of the composition of a microbial consortium selected in WP2A and its preparation for use in Saint-Nectaire and Pont L'Evêque cheese manufacturing. The initial consortium was composed of 11 lactic acid bacteria and 10 Gram + catalase + bacteria. A simplified consortium composed of 7 species of lactic acid bacteria belonging to the *Lactobacillus* (*Lb. casei*, *Lb. plantarum*, *Lb. curvatus*, *Lb. farciminis*) and *Leuconostoc* (*Ln. pseudomesenteroides*, *Ln. citreum*) genera and 7 species of Gram Catalase+ (*C. casei*, *C. flavescens*, *B. linens*, *B. rhamnosum*, *A. nicotianae*, *M. caseolyticus*, *S. equorum*) presented a similar level of inhibition against *L. monocytogenes* than the original consortium. This microbial consortium will be prepared for each farm assay in Saint-Nectaire cheese production and Pont L'Evêque production from frozen bacterial strains. The strains stored at -20°C will be mixed together in adequate concentrations the day before their use and stored at 4°C during one night until the inoculation in milk.

#### **Introduction**

The guaranty of cheese safety (inhibition of *Listeria monocytogenes*) without affecting their sensorial properties (flavour, aspect) is a major goal for traditional cheese producers. In this context, in task 2 of WP2A of the Truefood project, microbial consortia or strains with antagonistic activities have been studied in order to propose to small cheese producers a microbial consortium exhibiting an anti-*Listeria* activity. Among the studied antagonist consortia, one seemed particularly interesting. It has been defined by simplifying a complex consortium from raw milk with high anti-*Listeria* activities and its efficiency is similar to the complex consortium. It is composed of lactic acid bacteria, *Staphylococcus* and *Corynebacteria* able to inhibit the development of *Listeria* in the core of cheese or at the surface in our experimental ripening conditions (deliverable D22.10 about "*Final report on efficiency of strains or consortia for inhibiting pathogenic bacteria*"). The efficiency of this consortium was tested at an experimental pilot scale in an uncooked model cheese prepared with pasteurised milk. Before proposing this consortium to small cheese producers it is important to evaluate its easiness of use and its efficiency in real conditions (plant or farm production), and, moreover, to test its effect on the sensorial properties in small plant cheese production. In a first step, the composition of this consortium must be improved as some bacterial species may not fit with the recommendations of safety defined in the QPS concept (EFSA authority). Moreover, this consortium must be adapted to the two kinds of cheese chosen for application at industrial scale: raw milk Saint-Nectaire cheese manufactured in farms and a soft cheese Pont L'Evêque. It must be safe and easy to use.

#### **Objectives**

The objective was to improve the composition of a microbial consortium exhibiting anti-*Listeria* activities. The optimisation of the composition of this consortium and its preparation and condition of use are described in this deliverable.

## Optimisation of the composition of the consortium

### 1. Initial consortium

The initial anti-*Listeria* consortium isolated by Saubusse 2007 was composed of 32 species belonging to lactic acid bacteria (group A), Gram+ non-lactic acid bacteria (group B), Gram-bacteria (group C) and yeasts (group D). This consortium was successively simplified as described in task WP2A to evaluate which microbial populations or strains of the consortium obtained from raw milk are involved in the inhibition of *Listeria monocytogenes* in the core of cheese. It was concluded that the reduced consortium AB presented anti-*Listeria* properties similar to the complex consortium. This AB consortium (lactic acid bacteria and Gram+ non-lactic acid bacteria) obtained after all simplifications in WP2A is described in table 1.

Strains	
<i>Lb. casei</i> FH1 <i>Lb. curvatus</i> FH11 <i>Lb. paracasei</i> FH2 <i>Lb. plantarum</i> FH3 <i>Lb. farciminis</i> FH4 <i>Ln. citreum</i> MSE2 <i>Ln. pseudomeseteroides</i> MSE7	Group A Lactic acid bacteria
<i>St. saprophyticus</i> RPF10 <i>St. equorum</i> RPF6 <i>A. nicotianae</i> T9 <i>C. flavescens</i> CRBM7 <i>C. casei</i> CRBM9 <i>B. linens</i> CRBM12 <i>Exiguobacterium</i> sp. NL5 <i>St. haemolyticus</i> NL8 <i>Brachybacterium rhamnosum</i> PCA7 <i>M. caseolyticus</i> RPF8	Group B Gram + non-lactic acid bacteria

Table 1: Initial AB consortium before optimisation (WP2A)

### 2. Methodology

In this task WP6, this AB microbial consortium was optimised even more by omitting the species – *Staphylococcus saprophyticus*, *St. haemolyticus* and *Exiguobacterium* sp.- which may be considered as unsafe according to QPS recommendations.

In this way, a set of experiment was conducted in pasteurized uncooked pressed cheeses according to the same protocol than in the other sets of experiment (WP2A) (see protocol in deliverable D22.10 about “*Final report on efficiency of strains or consortia for inhibiting pathogenic bacteria*”).

Six assays were performed in parallel:

1. assay 1 = AB : all lactic acid bacteria of group A & all Gram + catalase + bacteria,
2. assay 2 =AB : *Lactobacillus* & *Leuconostoc* & all Gram + catalase + bacteria,
3. assay 3 = AB: *Lactobacillus* spp. & *Leuconostoc* spp. & B without *S. saprophyticus* and *S. haemolyticus*,

4. assay 4 = AB: *Lactobacillus* spp. & *Leuconostoc* spp. + B without *Exiguobacterium* sp.,
5. assay 5 = AB : *Lactobacillus* spp.& *Leuconostoc* spp. + B without *S. saprophyticus* and *S. haemolyticus* and *Exiguobacterium* sp.,
6. assay 6 = control inoculated with only *St. thermophilus*.

*L. monocytogenes* was enumerated in the core and at the surface of the cheeses according to the ISO112090-2 recommendations. The pH was measured in the core and at the surface of the cheeses after 1, 8, 18, 28 days of ripening.

## 2. Results

### a. In the core

The counts of *L. monocytogenes* in the core of cheeses (Table 2) varied according to the simplified consortia inoculated:

- The counts of *L. monocytogenes* in one day old cheeses were similar in all assays except in cheeses inoculated with leuconostocs and lactobacilli and bacteria of group B without *Exiguobacterium* sp.(assay 4) (0.5 to 0.75 log<sub>10</sub> units lower than the other assays).
- At 8 days of ripening, counts of *L. monocytogenes* in cheeses were the lowest for those inoculated with complex consortium AB (assay 1) or leuconostocs and lactobacilli as well as bacteria of group B except *Staphylococcus* spp. and *Exiguobacterium* sp.(assays 4 and 5) (1.4 to 1.8 log<sub>10</sub> units lower than the control cheese (assay 6)).
- At 18 days of ripening, the lowest counts of *L. monocytogenes* were observed in cheeses inoculated with only the species of the *Lactobacillus* and *Leuconostoc* genera complemented with all bacteria of group B (assay 2) (2 to 2.2 lower than the control cheese).
- At 28 days of ripening, the count of *L. monocytogenes* was the lowest and under the detection limit in cheeses inoculated with only the species of the *Lactobacillus* and *Leuconostoc* genera complemented with bacteria of group B except *Staphylococcus* spp. or *Exiguobacterium* sp. (assays 3, 4 and 5).

Whatever the ripening considered, the highest inhibition of *L. monocytogenes* was not associated with the highest pH , except for assay 3 at 8 days which had the highest counts and the highest pH.

### b. At the surface

At the surface of cheeses until 18 days all the reconstituted consortia were more inhibitory than the control. But the inhibition was not as strong as the ones observed in the core, associated with higher PH values. At 28 days, the consortium with leuconostocs and lactobacilli complemented with bacteria of group B except *Staphylococcus* spp. and *Exiguobacterium* sp. ( assay 5) was the most inhibitory (1.15 log<sub>10</sub> units lower than the control cheese ).

A: CORE OF CHEESES												
core	<i>Listeria</i>						pH					
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
1 day	3,23	3,14	3,34	<b>2,64</b>	3,49	3,39	5,4	5,44	5,44	5,4	5,45	5,41
8 days	<b>2,56</b>	3,2	3,56	2,92	2,95	4,32	5,28	5,27	5,32	5,27	5,27	5,32
18 days	2,34	<b>1,78</b>	2,9	2,7	<b>1,95</b>	3,95	5,16	5,08	5,14	5,08	5,06	5,19
28 days	1,7	1,6	<1	<1	<b>1</b>	2,23	5,07	5,1	5,06	5,09	5,1	5,19

B: SURFACE OF CHEESES												
	Listeria						pH					
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
1 day	2,87	3	3,59	3,28	3,46	3,73	5,63	5,64	5,63	5,64	5,6	5,66
8 days	3,2	3,8	3,9	3,8	3,9	4,97	6,36	6,47	6,23	6,85	6,76	6,86
18 days	3,85	3,68	3,99	3,74	3,7	4,64	6,01	6,81	7,08	7,08	6,93	6,98
28 days	3,49	3,81	4,1	3,38	<b>2,97</b>	4,11	6,62	7,09	7,29	7,31	7,07	7,11

Table 2. Counts of *Listeria monocytogenes* and pH values in A) the core of cheeses, B) at the surface inoculated with: assay 1 : all lactic acid bacteria of group A & all Gram + catalase + bacteria of group B, assay 2 : (*Lactobacillus* spp. + *Leuconostoc* spp.) & all Gram + catalase + bacteria of group B, assay 3 : (*Lactobacillus* spp. + *Leuconostoc* spp.) & B without *S. saprophyticus* and *S. haemolyticus*, assay 4 : (*Lactobacillus* spp. + *Leuconostoc* spp.) & B without *Exiguobacterium* sp., assay 5 : (*Lactobacillus* spp. + *Leuconostoc* spp.) & B without *S. saprophyticus* and *S. haemolyticus* and *Exiguobacterium* sp., assay 6 : control inoculated with only *St. thermophilus*.

### 3. Conclusions

The final consortium is composed of 7 species of lactic acid bacteria belonging to the *Lactobacillus* (*Lb. casei*, *Lb. plantarum*, *Lb. curvatus*, *Lb. farciminis*) and *Leuconostoc* (*Ln. pseudomesenteroides*, *Ln. citreum*) genera and 7 species of Gram+ Catalase+ bacteria (*C. casei*, *C. flavescens*, *B. linens*, *B. rhamnosum*, *A. nicotianae*, *M. caseolyticus*, *S. equorum*) . Compare to the original consortium (as described in table 1), the following species were omitted : *Staphylococcus saprophyticus*, *St. haemolyticus* and *Exiguobacterium* sp.

## Preparation of the consortium

### 1. Culture of strains and storage

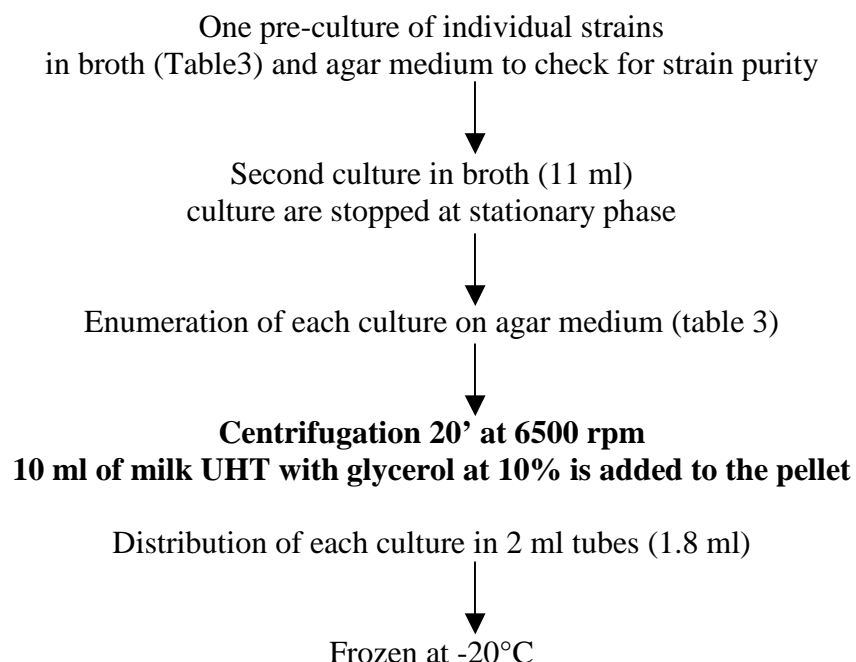


Table 3 : Final composition of consortium AB, culture and count media of strains and level of inoculation in milk.

culture media	Count media	level of inoculation in milk
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<i>Lb casei-paracasei</i>	MRS	FH	4,0
<i>Lb curvatus</i>	MRS	FH	3,0
<i>Lb plantarum</i>	MRS	FH	4,0
<i>Lb farciminis</i>	MRS	FH	3,0
<i>Ln pseudomesenteroides</i>	M17	MSE	3,0
<i>Ln citreum</i>	M17	MSE	3,0
<i>St. equorum</i>	BHI	BHI	2,5
<i>A. nicotiana</i>	BHI	BHI	3,0
<i>C. flavescens</i>	BHI	BHI	2,0
<i>C. casei</i>	BHI	BHI	2,0
<i>Br. Linens</i>	BHI	BHI	2,0
<i>Brachy. rhamnosum</i>	BHI	BHI	2,0
<i>M. caseolyticus</i>	BHI	BHI	2,0

All consortium strains have been sent by INRA to ADRIA Normandie and the same protocol of preparation and conservation of strains will be applied for the assays of validation in Farm Saint-Nectaire PDO Cheeses and in Pont-L'Evêque cheese .

## 2. Preparation of the consortium

As the lyophilisation's focusing of strains may be time consuming, it was chosen to use frozen cells.

The consortium will be prepared the day before the manufacturing (in the evening)

- One tube of 2 ml of each strain will be thawed at room temperature
- Adequate quantities of each strain for the final level (table 3) expected in milk (600L of milk) will be mixed in a sterile tube of 50 mL. The final level was defined according to the level of these species in milk ( Saubusse et al, 2007<sup>1</sup>)
- Then, the mix will be immediately cold stored on ice and transported to the farm or plant. It will be stocked at 4°C until the next day.

## 3. Inoculation in milk

- The consortium will be added to milk at the beginning of the milking at the same time that commercial bacterial starter culture used .

## 4. Cheese making

This consortium will be tested in:

- a. Saint-Nectaire : a small farm producing Saint-Nectaire raw milk cheese (non cooked pressed cheeses)
- b. Pont L'Evêque : a technological hall producing pasteurised Pont L'Evêque cheese (soft cheese).

### ▪ Farm Saint-Nectaire cheeses manufacturing and ripening

- The farm cheeses will be prepared as follow: raw milk, just after milking, will be inoculated with the usual starter culture (control) or with the usual starter culture added to the anti-*Listeria* consortium The control and the assay will not be prepared the same day but the same week (2 weeks of cheese-making with two or four productions per week). The farmer will prepare its cheeses as usual. Cheeses without *Listeria* will be ripened in the industrial

<sup>1</sup> Saubusse, M., Millet, L., Delbès, C., Callon, C., Montel, M.C. 2007. Application of Single Strand Conformation Polymorphism – PCR method to differentiate the cheese bacterial communities inhibiting *Listeria monocytogenes*. *International Journal of Food Microbiology*. 116 : 126-135

conditions (28 days at 9°C and near 98% hygrometry) in the same cellar than for task 2 of WP6 (Lanobre). At day one, 6 cheeses of each manufacturing will be transferred to INRA for *Listeria* inoculation and will be ripened in INRA cellar in 10°C at 97% of RH conditions of. 3 repetitions of manufacturing (control + assay with the consortium) will be performed.

▪ Pont L'Evêque manufacturing and ripening

For soft cheese, cheese production will be conducted in a small scale production in a technological hall. Production vats, each containing 60 l of pasteurized milk, will be inoculated either with the traditional Pont L'Evêque starter cocktail (control) or with the same starters supplemented with the anti-*Listeria* consortium (same levels of inoculation as those used for Saint-Nectaire). At day 2, the cheese will be salted; then, half of the control and test cheeses will be sent to the ACTIA-ADRIA Quimper partner for the *Listeria monocytogenes* inoculation. The other half will be kept in the technological hall and will follow the classical ripening Pont L'Evêque stages (At day 5, a smearing step then 10 days at 12°C and 98% of humidity followed by conservation step at 8°C up to 55 days). After inoculation with *L. monocytogenes*, the inoculated cheeses will follow the same process.

**Preparation and inoculation of *Listeria monocytogenes* at the surface of cheeses**

The strain SN 167 will be prepared and conserved in the same way that the consortium strains.

- Just before inoculation (on 1 day old cheeses for St-Nectaire technology and on 3 days old cheeses for Pont-L'Evêque technology), one tube of milk (1.8 ml) will be thawed at room temperature and then diluted in Ringer solution to obtain a final inoculation level of 2 to 5 CFU/cm<sup>2</sup>.

- Both cheese faces will be inoculated by depositing 3 mL of *Listeria* solution on all the surface and spreading using a sterile toothbrush.

**Analysis**

- Microbial, physical (pH, DM, Aw) and biochemical parameters will be analysed at 2, 8, 18 and 28 days of ripening for St-Nectaire cheeses and at the same times + 40 and 55 days for Pont-L'Evêque cheeses.

- *L. monocytogenes* will be enumerated at the surface of the cheeses inoculated according to ISO112090-2 recommendations at 1, 4, 8, 13, 18, 23 and 28 days in St- Nectaire and at 3, 5, 14, 19, 25, 31,38 and 53 days for Pont L'Evêque.

- At the end of ripening, the sensorial qualities of cheese (aspect, flavour) inoculated or not with the anti-*Listeria* consortium will be determined by sensory evaluation expert panels.