DETECTION BY *IN SITU* HYBRIDISATION OF MEAT STARTER CULTURE : *LACTOBACILLUS* AND *STAPHYLOCOCCUS*

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BACKGROUND :

Staphylococcus carnosus, Staphylococcus xylosus, Lactobacillus sake and *Lactobacillus curvatus* are used as starter culture in fermented sausages, contributing to flavour production and acidification. For controlling the quality of fermented meat products, it is necessary to estimate rapidly the specific bacterial count of starter culture inoculated and the natural flora : *Staphylococcus saprophyticus, Staphylococcus warneri, Staphylococcus epidermidis* and *Staphylococcus aureus*. The procedures based on counting colony-forming units (CFU) are times consuming and a specific identification need further biochemical tests. More recently, the development of *in situ* hybridisation techniques with fluorescently labelled rRNA-targeted nucleic acid, allows a rapid and specific *in situ* identification of individual cells in their natural habitats. The technique relies on the specific hybridisation of the nucleic acid probes to the naturally amplified intracellular rRNA (Amann *et al.*, 1995). Target sites of rRNA must then be specific and accessible. In this way direct detection is possible without the culture of micro-organisms and without amplification of nucleic acids. The technique has been successfully applied to the detection of *Pseudomonas* in soils (Hahn *et al.*, 1992), lactococci, streptococci in milk (Beimfohr *et al.*, 1993), *Holospora* in endosymbionts or magnetotactic bacteria in sediment (Amann *et al.*, 1995), Gram filamentous bacteria in sludge (Wagner *et al.*, 1994).

OBJECTIVES :

The aim of this work was to design specific probes for detecting *Staphylococcus* and *Lactobacillus* isolated from meat by *in situ* hybridisation and to apply this method to their detection in meat fermented product without cultivation.

MATERIALS AND METHODS :

Growth condition of the strains : Species of *Staphylococcus* were grown aerobically at 30°C in Brain Heart Infusion and species of *Lactobacillus* were grown aerobically at 30°C in *Lactobacillus* MRS broth.

Oligonucleotide probes : The oligonucleotide probes for *Staphylococcus* genus, *S. carnosus*, S. *warneri*, *S. saprophyticus, S. xylosus* and *Lactobacillus* were selected according to the alignment of 16S rRNA sequences from GenBank. The 23S rRNA oligonucleotide probe of *Lactobacillus sake* was determined by Hertel *et al.*, (1991). The specificity of the probe was tested on total rRNA by dot-blot hybridisation (Stahl *et al.*, 1988). Oligonucleotide probes used for *in situ* hybridisation were labelled with fluorochromes : tetramethylrhodamine or fluorescein.

Sample preparation : A meat fat mixture was inoculated with *S. carnosus* 833 and *L. sake* L110 and incubated 3 days at 22°C then at 13°C. At 30 days the mixture was diluted with saline solution (NaCl 8.5 g/l) and stomached 1 min. The supernatant was treated with enzyme trypsin at 0%, 2% and 10% and incubated 10 min at 50°C (Liberski, 1994). The sample treated with trypsin was filtered through a 10 µm polypropylene filter.

Enumeration of micro-organisms : Gram-positive catalase-positive cocci and lactic bacteria were enumerated using the Mannitol Salt Agar count and MRS agar count respectively. The analysis were performed before and after enzymatic treatment filtration.

In situ hybridisation : After enzymatic treatment, the mixture was centrifuged. Pellet was washed and fixed 30 min with paraformaldehyde solution (4%). Before being applied to gelatin coat microscopy slides, each sample was permeabilised 20 or 40 min with lytic enzymes : lysozyme (10 mg/ml) or lysostaphin (5 µg/ml). The procedure for the *in situ* hybridisation of whole cells with fluos-labelled oligonucleotide probes was described previously by Beimfohr *et al.* (1993).

RESULTS and DISCUSSION :

Oligonucleotide Probes :

Probes	specificity by dot-blot hybridisation	*in situ* Accessibility	hybridisation specificity
S-D-Eub- 0338-a-A-18*	*Eubacteria**	+*	*Eubacteria**
S-G-Staph -0462-a-A-23	ND	+	*Staphylococcus*
S-S-S.carno-0163-a-A-23	*S. carnosus*	-	
S-S-S.carno-0440-a-A-23	*S. carnosus*	+	*S. carnosus*
S-S-S.war-0180-a-A-23	*S. warneri*	+	*S. warneri*
S-S-S.sapro-0983-a-A-24	ND	+	*S. saprophyticus ; S. xylosus*
S-S-S.xylo-0180-a-A-24	ND	-	
S-G-Lb-0702-a-A-25	ND	+	*Lactobacillus*
L-S-L.sak-0154-a-A-21**	*Lactobacillus sake* **	-	

Table 1 : Specificity of oligonucleotide probes tested by dot blot and *in situ* hybridisation
ND : not done ; + accessible , - not accessible ; *(Amann, *et al.*, 1995) ; ** (Hertel, *et al.*, 1991)

The specificity of rRNA oligonucleotide probes was tested by dot blot with total rRNA and *in situ* hybridisation on pure culture of cells in exponential phase. *S. carnosus*, S. *warneri* and *L. sake* species targeted probes were determined specific by dot blot

hybridisation as shown in table 1. Unfortunately, not all these probes were not applicable to detect these species by *in situ* hybridisation. Indeed, one *S. carnosus* targeted-probe gave a signal after *in situ* hybridisation. *L. sake, S. carnosus* and *S. xylosus* targeted probes gave no signal by *in situ* hybridisation. No hybridisation could be due to target site accessibility by probe. Indeed, targeted 16S rRNA existed in a natural form and higher-order structures in the ribosomes, RNA-protein interaction should prevent probe hybridisation. Nevertheless Eubacteria targeted probe (Amann *et al.*, 1995) and *Staphylococcus, S. carnosus, S. warneri, S. saprophyticus* and *Lactobacillus* targeted oligonucleotide probes could be used for *in situ* hybridisation.

Sample Preparation : One difficulty encountered with *in situ* hybridisation in dry sausage, is the separation of micro-organism from meat and fat tissues. Also the final preparation needed to be relatively free from debris, the bacterial cells must be intact and clearly visible after hybridisation.

❶ Counting. Whatever the trypsin concentration, the bacterial count of Gram-positive catalase-positive cocci was similar before and after trypsin treatment (Table 2). But the filtration was not possible for the mixture no treated or treated with 0% and 2% trypsin. While 10% trypsin treatment digested protein matrix and permitted the rapid filtration, without attachment of bacteria.

❷ Microscopy. To control if trypsin treatment was effective for microscopy observation, samples treated with 0%, 2% and 10% trypsin were hybridised with S-D-Eub-0338-a-A-18 probes (Table 2). Observation of mixture treated with 0%, 2% trypsin showed bacteria bound to matrix. At 10% trypsin treatment, bacteria were detached of meat and tissues and after the filtration step debris were eliminated.

Trypsin concentration	Log cfu/g		Microscopy observation
	Gram+ catalase+ cocci	Lactic bacteria	after *in situ* hybridisation
☞ initial enumeration	7.95	8.15	
☞ 0%	7.69	ND	bacteria bound to meat
☞ 2%	8.20	ND	bacteria free and some bacteria bound to debris
☞ 10%	8.11	8.69	bacteria free
☞ 10% and filtration	8.3	9.6	bacteria free

Table 2 : Trypsin efficiency on enumeration and microscopy observation of mixture at 10 days

Permeabilisation of Cells : The cell wall of Gram positive bacteria must be permeabilised to allow the entry of the probes. The detection of *Staphylococcus* and *Lactobacillus* will only be possible after enzymatic treatment for partial digestion of the cell wall. *Lactobacilli* were permeabilised with lysozyme treatment 20 or 40 min, but no signal was observed after *in situ* hybridisation of *Lactobacilli* treated with lysostaphin. It could be due a lack of permeabilisation or a lysis. *Staphylococci* were only permeabilised with lysostaphin.. Consequently 2 enzymatic treatments were necessary to detect *Lactobacillus* and *Staphylococcus* species in dried sausages.

probes	detection	*in situ* hybridisation after Permeabilisation				
		without lytic enzyme	with lysozyme 10 mg/ml		with lysostaphin 3µg/µl	
			20 min	40 min	20 min	40 min
S-D-Bact-0338-a-A-18	*bacillus*	-	+++	+++	-	-
	cocci	-	-	+	++	++
S-G-Lb-0702-a-A-25	*Lactobacillus*	-	++	++	-	-
S-S-S.carno-0440-a-A-23	*S. carnosus*	-	-	-	++	+

Table 3 : Enzymatic treatment to detect *Staphylococcus* and *Lactobacillus* by *in situ* hybridisation in dry sausage
Hybridisation efficiency was evaluated by the fluorescence intensity of cells : [-] : No observed fluorescence, [+] poor fluorescent intensity , [++] good fluorescent intensity, [+++] strong fluorescent intensity

CONCLUSION :

The study has revealed the main difficulties for the detection *in situ* and how to overcome them. Before performing *in situ* hybridisation it is important to check if target sites are accessible. Indeed probes specific by dot blot were not always applicable to *in situ* hybridisation. 16S rRNA targeted oligonucleotide probes were now available to detect *Staphylococcus, S. carnosus* and *Lactobacillus* in dry sausage. The separation of micro-organisms from meat could be possible by using 10% trypsin. The limiting factor for *in situ* hybridisation will be the permeabilisation as there is no universal enzymatic treatment.
Further experiments are in progress to compare classic enumeration (CFU) and enumeration by *in situ* hybridisation coupled with an image analysis system.

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