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PRACTICAL APPLICATION OF IMMUNOFLUORESCENCE FOR THE DETECTION OF BACTERIAL CONTAMINANTS DURING VINIFICATION

UTILISATION D'UNE TECHNIQUE D'IMMUNOFLUORESCENCE POUR LA DÉTECTION DES BACTÉRIES LACTIQUES CONTAMINANTES EN VINIFICATION

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Abstract : This study presents a rapid and specific microscopic technique for detecting and identifying populations of lactic acid bacteria in musts, wines, and inoculum starter cultures. Through the use of fluorescent antibodies, this procedure can be performed in less than two hours, and it is effective with *Leuconostoc*, *Pediococcus* and *Lactobacillus* concentrations as small as 10^2 cells/ml. Implementation of this technique will assist winemakers in controlling malolactic fermentations and in preventing lactic acid bacterial spoilage.

Résumé : Ce travail présente une technique microscopique rapide et spécifique pour la détection et l'identification des bactéries lactiques dans les moûts, les vins ou dans une culture de levures ou de bactéries. Grace à l'utilisation de trois anticorps fluorescents, nous avons pu détecter la présence de *Leuconostoc oenos* en moins de deux heures avec des seuils de sensibilité de l'ordre de 10^2 bactéries/ml. L'étude de vins altérés a montré la fiabilité des réactifs sur les bactéries vivantes ou mortes, avec toutefois des bacilles non détectés en fluorescence dans un vin d'origine argentine. L'immunofluorescence s'est révélée comme une technique facile à mettre en oeuvre. Les méthodes plus sensibles de culture nécessitant plusieurs jours d'incubation avant la lecture du résultat, cette technique est aujourd'hui à considérer comme un outil d'aide à une décision technologique.

Mots clés : immunofluorescence, bactéries lactiques, détection, vin, moût

Key words : immunofluorescence, lactic acid bacteria, detection, wine, must

INTRODUCTION

The use of malolactic fermentation in winemaking is debated. Depending on must acidity and the final wine acid levels targeted, this bacterial fermentation may or may not be desired. Wine aroma and flavor changes resulting from this bacteriological process are also viewed subjectively. It is generally considered, however, to be beneficial in providing increased microbial stability.

In champagne making, the malolactic fermentation is extremely desirable. This fermentation generally occurs spontaneously, thanks to the *Leuconostoc*, *Pediococcus* and/or *Lactobacillus* bacteria that remain in the wine at the end of the alcoholic fermentation. The chemical characteristics of the wine that prevail at this stage are generally favorable to the development of these lactic acid bacteria genera. *Leuconostoc oenos* seems especially well-adapted for survival in Champagne base wines, and its growth presents no threat to wine quality.

In order to avoid growth and spoilage caused by indigenous populations, either of two inoculation methods can be used. A method developed by PARENTHOEN (1981) utilises refrigeration to reduce metabolism in wine lots where malolactic fermentation is well under way. The subsequent malolactic fermentations are rapid and the flavor and aroma of the wines produced are satisfactory. The second method involves the addition of commercial starter cultures of *Leuconostoc oenos*. Whatever the inoculating method, the purity of the starter culture is critical and must be devoid of undesirable genera.

In making wines from grapes harvested at higher maturity, the total preservation of malic acid may be desired. In this case, where the avoidance of bacte-

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TABLE I

Strains of bacteria used and their sources

Tableau I — Origine des souches de bactéries lactiques

Genus	Species	Clone	Source
Leuconostoc	*oenos*	G 7	Comité Interprof. des Vins de Champagne
Leuconostoc	*oenos*	E11	Comité Interprof. des Vins de Champagne
Leuconostoc	*oenos*	Inobacter	Institut Œnologique de Champagne
Leuconostoc	*oenos*	Viniflora	Chr. Hansen S.A.
Leuconostoc	*oenos*	MC1	Moët & Chandon
Lactobacillus	*plantarum*	ENSIA	ENSIA (École Nat. Sup. Indus. Agricoles)
Lactobacillus	not known	CIVC 3	Comité Interprof. des Vins de Champagne
Pediococcus	not known	CIVC 1	Comité Interprof. des Vins de Champagne

riological contamination is a primary goal, it would be useful to be able to rapidly control the purity of a yeast starter culture used for inoculation. Whether or not the malolactic fermentation is desired, the winemaker needs to be able to detect lactic acid bacteria and to determine if these bacteria are beneficial.

The aim of this work was to develop a specific method which could reveal quickly the presence of the three component genera of lactic acid bacteria in wine at different steps in the vinification process. Immunochemical techniques have the advantage of providing rapid results. Very few studies have been carried out on this subject. In the brewing industry, WHITING *et al.* (1992) describe the detection of *Pediococcus spp.* by an immunofluorescent technique on a membrane filter, and HUTTER (1991) studies immunofluorescence and tangential cytometry as applied to the detection of *Lactobacillus brevis* and *Pediococcus damnosus.* In the same way, RINCK and WACKERBAUER (1987) recommendes the use of immunofluorescent techniques for the detection of *Lactobacillus* in beer with an absorbed antiserum. The first work describing the detection of microbial contaminant by serological methods was carried out by DOLEZIL and KIRSOP (1975, 1976). THARRINGTON *et al.* (1978) show that unexpected cross-reactions exist between very different strains. We have not found any paper dealing with the serology of *Leuconostoc oenos*; however, LONVAUD-FUNEL *et al.* (1989) has advocated the identification of *Leuconostoc oenos* using DNA probes.

With wine bacteria, morphological differentiation can simplify interpretation. For this reason, indirect immunofluorescence, which BOUIX *et al.* (1988) applied for the detection of contaminants in musts during industrial fermentations, was chosen for this work.

MATERIALS AND METHODS

I — STRAINS OF BACTERIA AND YEAST (table I)

Most of the strains of lactic acid bacteria (LAB) used in this study were obtained from the Comité Interprofessionnel des Vins de Champagne (CIVC, Epernay, France). These LAB were isolated from grape juice from the Champagne area. Other strains of *Leuconostoc oenos* were commercial strains used in Champagne : Inobacter (Institut Œnologique de Champagne, Epernay, France) and Viniflora provided by Chr. Hansen France S.A. (Arpajon, France). Lastly the MC1 strain of *Leuconostoc oenos* was isolated and supplied by Moët et Chandon (Epernay, France).

Two commercial strains of *Saccharomyces cerevisiae* were used : Lalvin IOC (Institut Œnologique de Champagne) and Levulline CHP (Groupement des Laboratoires Oenologiques, Reuil-Malmaison, France).

II — CULTURE MEDIUM AND CULTURE CONDITIONS

The lactic acid bacteria were propagated in de Man-Rogosa-Sharpe (MRS) medium modified by the CIVC (MRS + 20 ml/l of centrifuged tomato juice + 5 g/l L-malic acid, pH 4.8). The cultures were grown at 30°C for 48 hours for *Lactobacillus* and *Pediococcus* and for 12 to 15 days for the *Leuconostoc* strains. Yeasts were propagated in a glucose Sabouraud's medium at 28°C for 24 or 48 hours.

III — SERA (table II)

Five anti-*Pediococcus* monoclonal antibodies, a mixture of ten anti-*Lactobacillus* monoclonal antibodies used in the dairy industry, three anti-*Lactobacillus* monoclonal antibodies and three anti-*Pediococcus* polyconal sera were supplied by Chemunex S.A. (Maisons-Alfort, France).

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TABLE II

Sera and monoclonal antibodies used and their sources

Tableau II — Origine des sérums et des anticorps monoclonaux

	Antibodies	Source
(A1)	anti-*Pediococcus* monoclonal antibody	Chemunex
(A2)	anti-*Pediococcus* monoclonal antibody	Chemunex
(A3)	anti-*Pediococcus* monoclonal antibody	Chemunex
(A4)	anti-*Pediococcus* monoclonal antibody	Chemunex
(A5)	anti-*Pediococcus* monoclonal antibody	Chemunex
(A7)	anti-*Lactobacillus* monoclonal antibody	Chemunex
(A8)	anti-*Lactobacillus* monoclonal antibody	Chemunex
(A9)	anti-*Lactobacillus* monoclonal antibody	Chemunex
(B)	anti-*Pediococcus* damnosus and pentosaceus	Chemunex
(CK7)	mixture of 10 anti-*Lactobacillus* monoclonal antibodies	Chemunex
(C)	anti-*Pediococcus* acidilactic polyclonal serum	Chemunex
(D)	anti-*Pediococcus* polyclonal serum	ENSIA
(E)	anti-*Leuconostoc oenos* G7 polyclonal serum	ENSIA
(F)	anti-*Leuconostoc oenos* E11 polyclonal serum	

The anti-*Leuconostoc* serum was prepared as follows. First, bacteria were cultivated for 12 to 15 days in modified MRS. The cells were then centrifuged and washed three times with sterile water. Rabbits were given injections of 10^8 cells at three ten-day intervals and a fourth booster injection seven weeks later. Two anti-sera were prepared for the two strains of *Leuconostoc oenos* G7 and E11.

The anti-yeast serum (Y) had already been used at the laboratory of microbiology of the Ecole Nationale Supérieure des Industries Agricoles et Alimentaires (ENSIA). It was an anti-*Saccharomyces cerevisiae* L226 serum (enological strain).

Sometimes a serum absorption (exhaustion) was necessary to eliminate a cross-reaction with a strain other than the immunisation strain. This was achieved by incubating 1 ml of serum with centrifuged and washed cells. The mixture was stirred for 6 hours at 37°C and stored at 4°C overnight. The cells were then removed by centrifugation. This process was repeated until a negative reaction with the initial strain was obtained.

Dilutions on sera were carried out depending on their titre. When called for, these dilutions were made with PGT (2.5 g Porcine Gelatin and 1 ml Tween 20 Sigma Chemical Co (St Louis, Missouri, USA) in 1 l physiological saline solution).

IV — IMMUNOFLUORESCENT TECHNIQUE

The indirect immunofluorescent technique was carried out using a serum conjugated with fluorescein isothiocyanate. The procedure is described in the following four steps.

1°) Fixation of the bacteria smear

Slide holes (Slide immuno CML L 10C7T, Teflon 2 x 5 holes D7 Red) were smeared with 20 µl of a suspension of micro-organisms (10^7 cells/ml for bacteria or 10^6 cells/ml for yeast). Subsequently, the smears were dried for 1 hour and fixed with ethanol. Slides were then saturated with 20 µl of PGT for 5 minutes at 37°C in a moist atmosphere.

2°) Antibody incubation

Following fixation, slides were smeared with 20 µl of PGT-diluted serum or monoclonal antibody, incubated for 20 minutes at 37°C in a moist atmosphere, and washed with PGT.

3°) Fluorescent conjugate incubation

The conjugate was diluted (1/5000) with a PGT solution containing Evans blue (1 g/l, Sigma E 2129) to color yeast or with a PGT solution containing propidium iodide (10 mg/l, Sigma P 4170) to color bacteria. The PGT and conjugate solutions were filtered (0.45 µm) with disposable syringe filters (Sartorius Minisart NML). Slides were smeared with 20 µL of a diluted, fluorescent conjugate: anti-mouse for the monoclonals (Ac Anti IgG (H+L) Souris Biosys (Compiègne, France) FITC 2 mg/ml, BI2113CE/2239) and anti-rabbit for the sera (Ac Anti IgG (H+L) Lapin Biosys FITC 4 mg/2 ml, BI2107/2097). The slide was incubated for 20 minutes in a moist atmosphere at 37°C, rinsed with

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PGT and air-dried. The smear was then mounted with a drop of buffered glycerol (0.136 g KH_2PO_4 ; 3.221 g Na_2HPO_4, $12H_2O$; 20 ml H_2O ; 80 ml (pH 8-9) glycerol) and with a cover slip (Lamelle immuno CML Diament star LDS 24-60).

4°) Microscopic observation

The slides were examined at 1250X using a oil immersion microscope (Leitz Dialux 20) fitted with an FITC blue light episcopic fluorescent attachment. Cutoff filters were used to select the green light emitted by fluorescein and the red light emitted by Evans blue. Positively reacting bacteria appeared fluorescent green with their cell walls even more intensely colored. The intensity of the fluorescence, was noted as: extreme (+++), very (++), somewhat (+), and not at all (-). Negatively reacting cells were colored red or pink by Evans blue or propidium iodide.

V — DETERMINATION OF THE SENSITIVITY THRESHOLD

1°) Detection of bacteria in a yeast suspension

To replicate the conditions of both a must during the primary fermentation and a sparkling wine yeast starter culture (developed in wine for bottle fermentation), yeast (Levulline CHP) were suspended in both grape juice and in wine to obtain approximately 3 x 10^8 cells/ml. Five bottles containing the must or the culture suspension, were experimentally contaminated with bacteria at concentrations ranging from 10^2 to 10^6 cells/ml.

In order to prevent high yeast cell concentrations from perturbing examinations, portions of the yeast were removed by mild centrifugation (9). Different speeds and durations were tested : 2 min at 20 g, 2 min at 45 g, 2 min at 85 g, 3 min at 90 g, 2 min at 200 g. The enumeration of bacteria in the supernatant was determined by direct epifluorescence technique (DEFT) after filtration of 20 ml through 0.2 µm-pore-size membrane filters and coloration with acridine orange. Twenty microscopic fields were observed to permit an approximation of the population of bacteria in the supernatant. The loss of bacteria could be estimated to about 10 p. cent and considerated as no important. After this initial centrifugation, 100 ml of the supernatant was centrifuged for 10 min at 6000 g to pellet the bacteria. Subsequently, the supernatant was decanted and the bacteria were resuspended in 0.5 ml of sterile physiological saline solution (9 g/l NaCl). Slide smears of these five samples enabled determination of the detection thresholds of bacteria in a yeast suspension.

2°) Detection of *Lactobacillus* and *Pediococcus* in a *Leuconostoc oenos* culture

A concentrated *Leuconostoc oenos* suspension was prepared with Inobacter containing approximately 10^8 cells/ml. Quantities of this suspension were then contaminated with cultures of *Lactobacillus* and *Pediococcus* suspended in MRS medium in order to obtain a contamination concentration of 10^2 to 10^6 cells/ml. Slide smears were made from these solutions.

3°) Contamination control of commercial products

Purity tests of the commercial strains of MC1, Viniflora and Inobacter were carried out after three days of growth in an MRS medium. This rapid culture process amplified the possible contamination of *Lactobacillus*, which grows quickly in this medium, without modifying the concentration of *Leuconostoc*, which does not.

4°) Control of the population present in a sample of base wine

Smears were made either directly from the base wines or following a centrifugation step which concentrated the bacteria. In the latter case, the supernatant was decanted, and the bacteria were then suspended in sterile saline water.

5°) Control of purity in a yeast starter culture

Samples were moderately centrifuged (200 g for 2 min) to eliminate yeast and produce supernatants to be used in immunofluorescent observation.

RESULTS AND DISCUSSION

I — REACTANTS

1°) Study of serum reaction with the homologous strain (table III)

Detection of *Pediococcus* CIVC 1 (undetermined species) : None of the five monoclonal antibodies (A1, A2, A3, A4, A5) revealed the strain *Pediococcus* CIVC 1. On the other hand, a fluorescence did appear on bacteria cell walls with the anti-*Pediococcus damnosus* and *pentosaceus* (B) polyclonal serum, the anti-*Pediococcus acidilactici* serum (C) and with the anti-*Pediococcus* mixture (D) (figure 1). This fluorescence was very intense with sera (B) and (D) and less so with serum (C).

Detection of two *Lactobacillus* : *Lactobacillus* (undetermined species) CIVC 3 (figure 2) and *Lactobacillus plantarum* ENSIA (figure 3) reacted strongly with the mixture of monoclonal antibodies (CK7). The response was weaker with monoclonal

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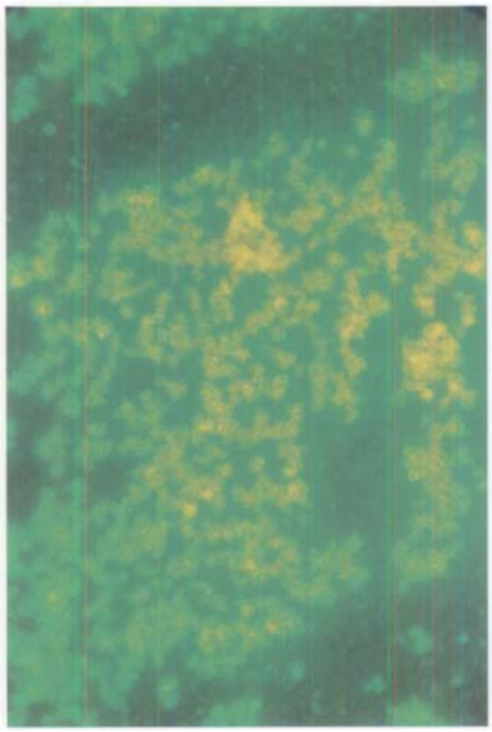


Fig. 1 — *Pediococcus* CIVC 1 revealed by the serum (D)

Fig. 1 — *Pediococcus* CIVC 1 révélé avec le sérum (D)

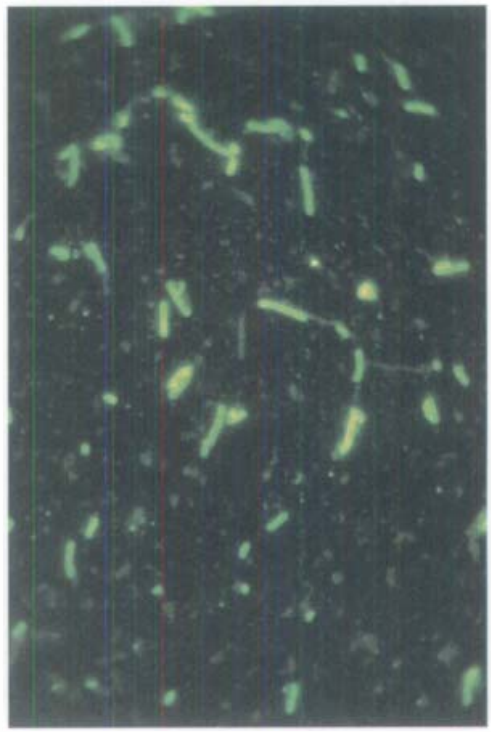


Fig. 2 — *Lactobacillus* CIVC 3 revealed by the mixture of 10 monoclonal antibodies (CK7)

Fig. 2 — *Lactobacillus* CIVC 3 révélé avec le mélange de 10 anticorps monclonaux (CK7)

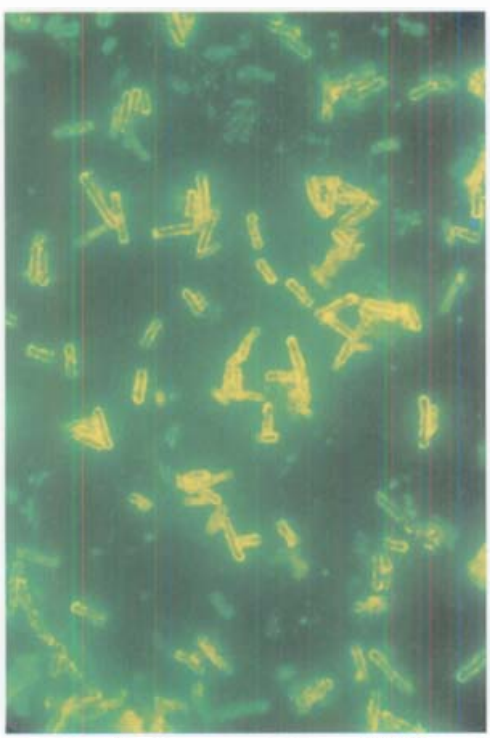


Fig. 3 — *Lactobacillus plantarum* revealed by the mixture of 10 monoclonal antibodies (CK7)

Fig. 3 — *Lactobacillus plantarum* révélé avec le mélange de 10 anticorps monclonaux (CK7)

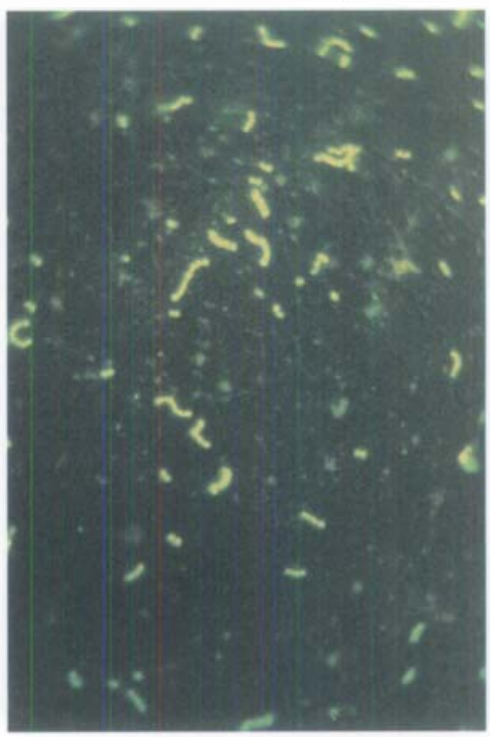


Fig. 4 — *Leuconostoc oenos* G7 revealed by the serum (E)

Fig. 4 — *Leuconostoc oenos* G7 révélé avec le sérum (E)

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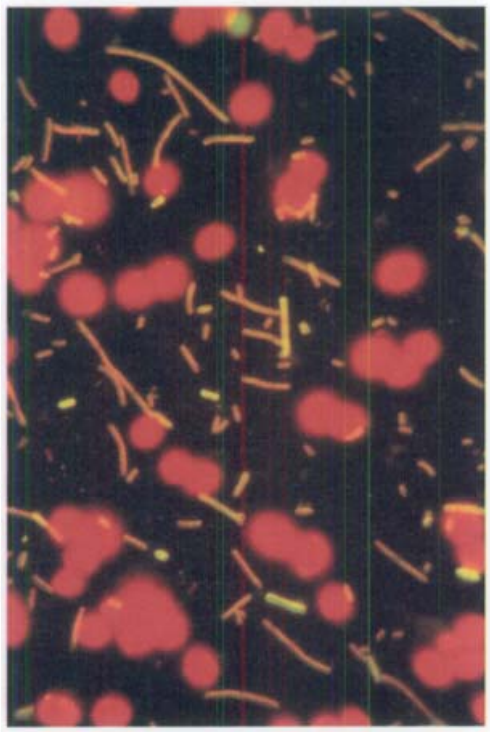


Fig. 5 — *Lactobacillus* CIVC3 revealed among yeast by the mixture of 10 monoclonal antibodies (CK7)

Fig. 5 — *Lactobacillus* CIVC3 révélé parmi des levures avec le mélange d'anticorps monclonaux (CK7)

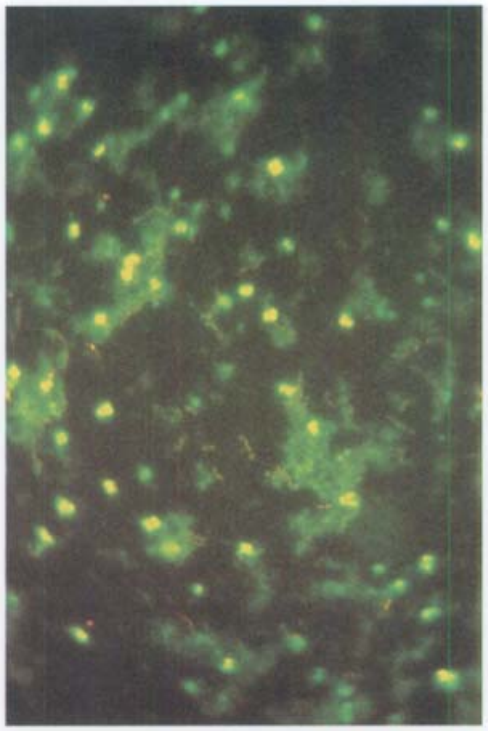


Fig. 6 — *Pediococcus* CIVC1 revealed among *Leuconostoc oenos* by the serum (D) after exhaustion

Fig. 6 — *Pediococcus* CIVC1 révélé parmi des *Leuconostoc oenos* avec le sérum (D) après épuisement

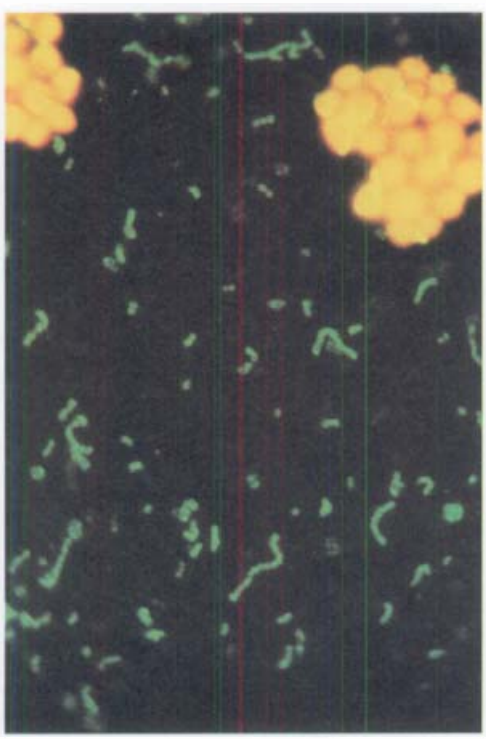


Fig. 7 — *Leuconostoc oenos* revealed among yeast cells by the serum (E) after exhaustion

Fig. 7 — *Leuconostoc oenos* révélé parmi les levures avec le serum (E) épuisé

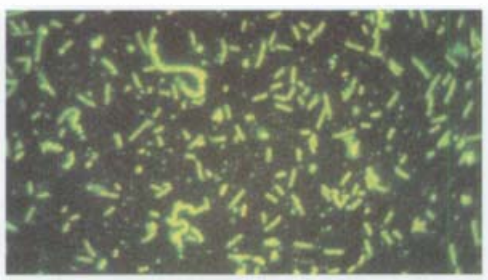


Fig. 8 — *Leuconostoc oenos* revealed among *Lactobacillus* by serum (E) after exhaustion

Fig. 8 — *Leuconostoc oenos* révélé parmi des *Lactobacillus* avec le sérum (E) épuisé

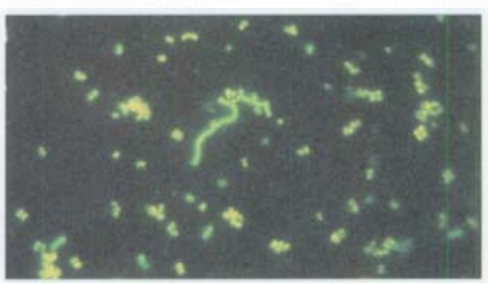


Fig. 9 — *Leuconostoc oenos* revealed among *Pediococcus* by serum (E) after exhaustion

Fig. 9 — *Leuconostoc oenos* révélé parmi des *Pediococcus* avec le sérum (E) épuisé

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TABLE III

Reactions of monoclonal antibodies and polyclonal sera with the Champagne wine strains of lactic acid bacteria

Table III — Réactions des anticorps monoclonaux et des sérums polyclonaux avec des souches de bactéries lactiques isolées dans les vins de Champagne

	anti-*Pediococcus* reactants							
	Monoclonal antibodies					Sera		
	(A1)	(A2)	(A3)	(A4)	(A5)	(B)	(C)	(D)
Pediococcus CIVC 1 undetermined species	-	-	-	-	-	++	+	++
	anti-*Lactobacillus* reactants : Monoclonal antibodies							
	(CK7)		(A7)		(A8)		(A9)	
Lactobacillus CIVC 3 undetermined species	+++		-		±		-	
	anti-*Leuconostoc* reactants : Sera							
	(E)				(F)			
Leuconostoc oenos G7	++				±			
Leuconostoc oenos E11	++				+			
Leuconostoc oenos MC1	++				++			
Leuconostoc oenos Inobacter	++				++			

antibody (A8) and practically non-existant with monoclonal antibodies (A7) and (A9).

Detection of *Leuconostoc oenos* : The first anti-*Leuconostoc oenos* G7 and anti-*Leuconostoc* E11 polyclonal sera, produced after the initial series of immunisations, enabled an intermediate fluorescence to be obtained. After some booster injections, the sera reacted well with the *Leuconostoc oenos* G7 (figure 4), E11 and with the *Leuconostoc oenos* Inobacter. The serum anti-*Leuconostoc oenos* G7 (E) was determined to be superior to the anti-*Leuconostoc oenos* E11 (F).

For the continuation of this work, we selected polyclonal serum (D), monoclonal antibody mixture (CK7) and polyclonal serum (E) to detect *Pediococcus* CIVC 1, *Lactobacillus* CIVC 3 and the *Leuconostoc oenos* species respectively.

2°) Cross-reactions with the primary sera (table IV)

The mixture of monoclonal anti-bodies (CK7) produced no reaction with strains of the genera *Pediococcus*, *Leuconostoc* or the yeast. On the other hand, (CK7) did cause a strain of *Lactobacillus plantarum* to become fluorescent. Fluorescence results were negative with all the other tested lactic acid bacteria.

The anti-*Pediococcus* polyclonal serum (D) did not react with *Lactobacillus* CIVC 3 and *L. plantarum* ENSIA. On the other hand, it did react strongly with all the *Leuconostoc oenos* strains and with yeast. As a result, it was very difficult to detect *Pediococcus* contaminants in *Leuconostoc oenos* starter cultures, yeast starter cultures, and in musts.

The anti-*Leuconostoc oenos* G7 polyclonal serum (E) reacted weakly with *Lactobacillus plantarum*, but strongly with yeast, *Pediococcus* CIVC 1 and *Lactobacillus* CIVC 3.

The detection of a single green, fluorescent bacterium in a microscopic field containing a large number of fluorescent yeasts and/or bacteria was very difficult, but that of one among invisible or alternately colored micro-organisms was easier. In order to detect a microbial spoilage or to identify a *Leuconostoc oenos* in a suspension like wine or must, the anti-*Leuconostoc* serum must not be allowed to react with the genera *Lactobacillus*, *Pediococcus* or yeast. In order to eliminate the antibodies corresponding to the common antigens of *Leuconostoc* and yeast, and to avoid cross-reactions, the crude anti-*Pediococcus* (D) serum was exhausted with pure yeast and *Leuconostoc oenos* cultures. In the same way, the serum (E) had to be exhausted to become specific to only the *Leuconostoc* species.

The (CK7) was shown to react with *Lactobacillus* CIVC 3 and *Lactobacillus plantarum* ENSIA.

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As the similarity of antigens between *Leuconostoc oenos* and *Saccharomyces cerevisiae* was unexpected, an anti-*Saccharomyces* serum procedure was used to verification. This serum, which strongly reacted with champagne yeast, revealed a fluorescence with *Leuconostoc oenos* which confirmed the antigenic similarity between the two types of micro-organisms.

3°) Exhausted sera study

Following exhaustions of the anti-*Pediococcus* serum (D) with *Leuconostoc oenos* Inobacter or *Saccharomyces cerevisiae* CHP, the serum still reacted with *Pediococcus* (table V). The anti-*Pediococcus*-*Leuconostoc*-exhausted serum, however, was still positive with yeast and the anti-*Pediococcus*-yeast-exhausted serum still reacted with *Leuconostoc*. Therefore, as it was shown that the two different antibodies were eliminated by two different absorptions, a double absorption (yeast and *Leuconostoc*) was necessary to obtain a serum specific to *Pediococcus* (table VI). In the same way, three absorptions were necessary in order to obtain a serum (E) specific to *Leuconostoc*.

TABLE IV

Cross-reactions of polyclonal sera (B), (D), (E) and (Y) and of monoclonal antibodies mixture (CK7) with genera of lactic acid bacteria and with yeasts

Tableau IV — Réactions croisées entre les sérums polyclonaux (B), (D), (E) et (Y) ou le mélange d'anticorps monoclonaux (CK7) et différentes espèces de bactéries lactiques ou des levures

	(B) anti-*Pedio.*	(D) anti-*Pedio.*	(CK7) anti-*Lacto.*	(E) anti-*Leuco.*	(Y) anti-*yeast*
L.A.B.					
Pediococcus CIVC 1 undetermined species	++	++	-	++	
Leuconostoc oenos G7		++	-	++	
Leuconostoc oenos E11		++	-	++	
Leuconostoc oenos MC1		++	-	++	++
Leuconostoc oenos Inobacter		++	-	++	++
Lactobacillus CIVC 3 undetermined species	+	-	+++	++	
Lactobacillus plantarum ENSIA	+	-	++	±	
YEAST : *Saccharomyces cerevisiae*					
Levulline		++	-		+++
CHP		++	-	++	+++
Lalvin IOC		++	-		+++

TABLE V

Fluorescence reactions after serum exhaustions

Table V — Réactions de fluorescence après absorption simple

Serum	(D)	(D)	(E)	(E)	(E)
Microorganism used for exhaustion	*Leuconostoc* Inobacter	Yeast	*Pediococcus*	Yeast	*Lactobacillus*
Microorganism tested for :					
Pediococcus CIVC1 undetermined species	++	++	-	++	+
Lactobacillus CIVC3 undetermined species			++	++	-
Leuconostoc oenos Inobacter	-	+	++	++	++
Saccharomyces cerevisiae CHP	++	-	+++	-	+++

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TABLE VI

Fluorescence reactions after multiple absorptions

Table au VI — Réactions de fluorescence après plusieurs absorptions

Serum	(D)	(E)	(E)	(E)	(E)
Microorganism used for exhaustion	*Leuconostoc* Inobacter and yeast 783	*Pediococcus* and *Lactobacillus*	*Lactobacillus* and yeast	*Pediococcus* and yeast	*Lactobacillus Pediococcus* and yeast
Microorganism tested for :					
Pediococcus CIVC1 undetermined species	++	-	+	-	-
Lactobacillus CIVC3 undetermined species	-	-	-	-	-
Leuconostoc oenos Inobacter	-	++	++	++	++
Yeast 783	-	+++	-	-	-
New name after exhaustion	(D')				(E')

TABLE VII

Detection threshold by direct observation of a yeast suspension (3×10^8 cells/ml) artifically contaminated by lactic acid bacteria

Tableau VII — Seuil de détection par observation microscopique directe de la contamination d'une suspension de levures (3×10^8 levures/ml) par des bactéries lactiques

Level of contamination (cells/ml)	10^6	10^5	10^4	10^3
Lactobacillus revealed by (CK7)	+	+	+ rare	-
Pediococcus revealed by (D) abs. CHP	+	+	+ rare	-
Leuconostoc revealed by (E) abs. CHP	+	+	+	-

(+) detection ; (-) absence of a fluorescent cell in several fields ; (rare) less than one fluorescent bacterium per field
(+) présence de cellules fluorescentes ; (-) absence de cellule fluorescente dans plusieurs champs
(rare) moins d'une bactérie fluorescente par champ

It is seen that the mixture of monoclonal antibodies (CK7) permitted easy detection of *Lactobacillus plantarum* ENSIA and *Lactobacillus* CIVC 3 among yeast and *Leuconostoc oenos* (figure 5). Figure 6 shows that detection with serum (D) following exhaustions with both yeast CHP and *Leuconostoc oenos* Inobacter enabled detection of *Pediococcus* CIVC 1 among *Leuconostoc oenos* cells. This serum was named (D'). The serum (E), after exhaustions with *Lactobacillus* CIVC 3, *Pediococcus* CIVC 1 and yeast CHP, enabled both detection of *Leuconostoc oenos* among yeast (figure 7) and easy differentiation among *Lactobacillus* (figure 8) and *Pediococcus* (figure 9). This serum was named (E').

II — SENSITIVITY THRESHOLD

The detection threshold of the three bacterial genera in a suspension of yeasts in water (3×10^8 cells/ml) is approximately 10^3-10^4 cells/ml (table VII). In grape juice or in fermenting must artificially contaminated by *Leuconostoc oenos* Inobacter, the detection was more difficult than in water because of the presence of particles in suspension which hid the bacteria. Moreover, the fixation of microrganisms on the examination slides was made more difficult in sweetened media because the cells detached during rinsing. The bacteria detection threshold was 10^5 cells/ml for grape juice and 10^6 cells/ml for fermented must. Mild centrifugations improved this threshold. The ideal conditions for centrifugation were two minutes at 200 g ; this allowed for the clarification of the suspension of yeasts without significantly modifying the concentration of bacteria. Table VIII shows the bacteria detection threshold by immunofluorescence with the sera. The wine used here contained yeasts at 3×10^8 cells/ml ; it was centrifuged initially for 2 minutes at 200 g to eliminate the yeasts and then for 10 minutes at 6000 g to concentrate the bacteria. The detection threshold in the treated wine was about 10^2 cells/ml for *Lactobacillus* and *Leuconostoc*, and 10^3 cells/ml for *Pediococcus*.

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TABLE VIII

Evaluation of the detection threshold of a yeast suspension (3 x 10^8 cells/ml) contaminated by lactic acid bacteria, after a mild centrifugation (2 min at 200 g), recuperation of the supernatant, and subsequent centrifugation (10 min at 6000 g)

Tableau VIII — Évaluation du seuil de détection de la contamination d'une suspension de levure (3 x 10^8 levures/ml) par des bactéries lactiques après centrifugation (2 min à 200 g) et récupération du surnageant pour une concentration par centrifugation (10 min à 6000 g)

Level of contamination (cells/ml) before centrifugation	10^4	10^3	10^2
Lactobacillus revealed by (CK7)	+	+	rare
Pediococcus revealed by (D) abs. CHP	+	rare	-
Leuconostoc revealed by (E) abs. CHP	+	+	rare

(+) detection ; (-) absence of a fluorescent cell in several fields ; (rare) less than one fluorescent bacterium per field
(+) présence de cellules fluorescentes ; (-) absence de cellule fluorescente dans plusieurs champs ; (rare) moins d'une bactérie fluorescente par champ

The detection threshold of *Lactobacillus* and *Pediococcus* in a suspension of *Leuconostoc*, normally 10^3 cells/ml, improved to 10^2 cells/ml with a 10-minute centrifugation at 6000 g and a resuspension to a smaller volume which concentrated the bacteria before microscopic preparation.

III — PRODUCT CONTROL APPLICATIONS

Where presumption of contamination existed, the commercial strains, inoculum and wines, were analysed with the three reactants according to the following described procedures.

1°) Control of dry, commercial malolactic bacteria

Samples of *Leuconostoc oenos* MC1, Inobacter and Viniflora were used. Observation with the (CK7) reactant produced negative results and the samples were deemed not to be contaminated by *Lactobacillus*.

2°) Control of an industrial malolactic bacteria culture

The industrial starter culture (MC1 *Leuconostoc*) was reactivated in young wine prior to use. Direct microscopic observation of the wine showed the presence of yeasts. After centrifugation at 200 g for 2 minutes, observation by immunofluorescence with the (CK7) reactant enabled detection and identification of some *Lactobacillus* cells. Since the purity of the strain of *Leuconostoc oenos* had been previously verified, this contamination probably was present in the non-sterilized wine before it was inoculated.

3°) Control of an industrial yeast culture

In order to avoid the contamination of musts and sparkling base wines with malolactic bacteria at the time of yeast inoculation, the immunofluorescent technique could be used to verify the absence of bacteria in the yeast culture. The yeast culture used in this portion of our study (5 x 10^7 cells/ml) was developed and stored in a sparkling base wine. After a first mild centrifugation of the culture and a subsequent centrifugation of the supernatant (10 min at 6000 g) and resuspension to 0.5 ml, observation by indirect immunofluorescence produced a very low detection threshold of approximately 10^2 cells/ml of original sample. This level represents less than one bacterium per microscopic field at 1250X magnification.

4°) Identification of bacteria genera in spoiled wines

Two bottled wines presenting abnormal organoleptic characteristics were analysed with the anti-*Leuconostoc* (E') sera, and the anti-*Lactobacillus* antibody mixture (CK7). A third bottled wine was analysed with the sera (E'), (CK7) and the anti-*Pediococcus* (D') serum

The first wine analysed was a turbid, two-year-old red Burgundy. Routine microscopic observation of this wine showed the presence of *bacillus*-like and *coccus*-like bacteria, and yeast. Slide preparations were made following wine centrifugation at 4000 g for 10 minutes and three subsequent washings with a saline solution. Observation by immunofluorescence with the (CK7) reactant revealed the presence of *Lactobacillus* while those with the (E') reactant did not reveal the presence of *Leuconostoc*.

The second wine studied was a turbid, red wine from Argentina which had suffered a halt in the alcoholic fermentation, and had a strong odour of acetic acid. Simple microscopic observation of this wine showed the presence of yeast, bacillus-like and coccus-like bacteria. Immunofluorescence enabled identification of *Leuconostoc* with the (E') serum, but the bacilli

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were not fluorescent with the (CK7) reactant. The failure of the (CK7) monoclonal mixture to identify the *bacilli* might have demonstrated that the monoclonal antibody mixture didn't have a spectrum sufficiently large to enable detection of all *Lactobacilli*. It was also possible that the *bacilli* observed with direct microscopic observation did not belong to the genus *Lactobacillus*.

The last wine analyzed, also red and turbid, displayed various abnormal aromas. Direct microscopic observation revealed the presence of yeasts, *bacilli*-like and *cocci*-like (tetrads and chains) bacteria. A mild 2-minute centrifugation at 200 g was performed before placing the smear on the slide. The chains were fluorescent with the (E') serum which indicated that they were of the *Leuconostoc* genus. In contrast, the tetrad cocci reacted positively with the (D') serum only, indicating that they belonged to the *Pediococcus* genus. Finally, the *bacilli* were fluorescent with the (CK7) mixture which identified them as *Lactobacilli*.

Immunofluorescent analysis of these wines was able to detect and identify in most cases both living and dead spoilage bacteria that were not identifiable by direct microscopic observation.

CONCLUSION

This immunofluorescent technique has been shown to produce rapid results when testing for culture purity of yeast or bacterial starter cultures and for must or wine contamination. Using the three reactants (D'), (E') and (CK7), it was possible to obtain detection threshold concentrations of 10^2 cells/ml for *Leuconostoc oenos* and *Lactobacillus* and 10^3 cells/ml for *Pediococcus* in yeast starter cultures, musts and wines. In all but one case, that of a *bacillus* in the Argentinian red wine, analysis of deteriorated products also demonstrated the effectiveness of the reactants in correctly identifying the genera of both living and dead bacteria.

The effectiveness of this immunochemical technique is very dependent upon the antibodies contained in the reactants. Monoclonal antibody reactants were in some cases too specific in light of the diversity of the microbial populations found in fermenting wines. In order to detect all the strains of a bacteria genus, for example, it was necessary to use a mixture of antibodies to extend the response spectrum. The Argentinian wine analysis, in which a ten-antibody mixture failed to detect a *bacilli* thought to be seen in prior microscopic observation, demonstrated that mixtures may need to include even greater numbers of specific antibodies.

Polyclonal sera, on the other hand, may possess detection spectra that are too large to be specific. As there are numerous antigenic similarities between wine microorganisms, use of polyclonal sera can produce reactions with both yeast and the different genera of lactic acid bacteria. In order to control these cross reactions, sera must be exhausted with the potentially interfering microorganisms to improve their specificity for the bacteria being tested for.

Present lack of knowledge regarding the antigenic structure of wine micro organisms makes it impossible to guarantee absolute accuracy with this identification method. Nevertheless, the presented immunofluorescent technique, which combines morphological observation with an immunochemical response, does permit reactant specificity for genera of wine bacteria.

An important shortcoming of the present technique is that its detection threshold (10^2 cells/ml) may not be small enough for all cases. If greater sensitivity is desired, the procedure might be modified by using greater volumes of clear product only, and by carrying out the immunochemical reaction directly on the filter membrane.

The greatest advantage of this immunofluorescent bacterial detection technique is that it can be conducted rapidly, allowing results to be obtained in only two hours. More sensitive culture methods do exist, but they require several days or weeks to carry out, and are only effective with live cells. We conclude that the presented method be considered as only one part of a technological decision support system.

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