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NEWSLETTER

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PSEUDOMONAS SPP ENUMERATION IN DAIRY PRODUCTS : DIFFICULTY TO CHOOSE AN APPROPRIATE METHOD

PART 1 : BIBLIOGRAPHIC REVIEW AND PRESENTATION OF EXPERIMENTAL STUDY

Within the context of the analysis methods standardization, ISO proposed a horizontal method for the *Pseudomonas* SPP enumeration in food products and animal feeding (ISO/WD 13720). But the relevance of this method in milk products arises interrogation. A bibliographic and technical study was carried out in order to give brief replies. This study is organized around an inventory of enumeration methods described in the scientific literature and of the methods set up by French milk payment laboratories. It relates to the description of the *Pseudomonas* or not *Pseudomonas* flora most frequently counted by the methods mentioned above. Finally it relates to a quantitative and qualitative comparison of the enumeration values obtained according to 3 protocols (of which that proposed by standard ISO/WD 13720) for 60 samples of cheeses or cheese-making specialities.

PSEUDOMONAS SPP IDENTITY CARD

They gather more than 140 species that have a common high capacity for adaptation and the following characteristics :
Bacilli with negative Gram of 0.5-1.0 x 1.5-5.0 µm, generally motile thanks to one or more polar flagella. As aerobic germs, they have an oxidative metabolism but some of them can use nitrates as final electron acceptors in anaerobic conditions. Most of them do not require organic growth factors ; and are chemo-organotrophic, but some of them can be optional chemo-lithotrophic and use H_2 ou CO_2 as source of energy. Lastly, they can be oxydase+ or oxydase- but always catalase+.

PROBLEMS AND DEFECTS OCCURED BY *PSEUDOMONAS* SPP

In the dairy sector, the emergence of *Pseudomonas* spp raises a major problem ; that concerns the whole of actors :
- producers, transformers and ripeners - and potentially all the categories of dairy products, whatever the nature of milk – raw milk or heated milk – or its origin – cow or goat or sheep milk.
Those micro-organisms that constitute until 60 % of the psychrotrophic flora in raw milk are likely to harm the good course of the product manufacturing (material fouling, clotting defects, clotting times mofified, lack of firmness of curds, low cheese yield) and even induce deteriorations of the organoleptic quality leading to the down grading with the rejection of the products. Damaging results from several phenomena :

- the production of mucus substances which induces smearing on the cheeses surface and limits the maturing flora, probably through joint productions of antifungic metabolites,
- the production of lipolytic and proteolytic enzymes which leads to major organoleptic defects for all types of manufactured products because these enzymes have the particular capability to resist to heat treatments. Casein and lipids degradation causes many defects in cheese texture (softened paste) but also in flavour such as bitterness, rancidity or cardboard taste,
- the synthesis of pyrazin type metabolites with a very weak smell, which gives contaminated cheeses particularly unpleasant potato odours
- the siderophores production (such as green-fluorescent-pyoverdine) which leads to modifications of the microbial ecosystems of cheese and its yellow to brown colouring.

SOURCES AND VECTORS OF CONTAMINATION BY *PSEUDOMONAS* SPP

Pseudomonas spp are regarded as ubiquitous germs, they frequently colonize water, grounds and more frequently the rhizosphere. In variable initial concentration in milk, they can contaminate the products at multiple stages of manufacture later on. Their most often declared origins are milk (via the colonization of surfaces), water (of food or cleaning station recycling), surfaces (opened or closed) and environment. Thereafter, within the complex matrices that constitute cheese products, they are subjected to variable and often sub-lethal environmental conditions such as high temperatures, low oxygen concentrations, or low AW and pH values associated with the presence of competitor flora.

The candidates of enumeration are thus *Pseudomonas* spp of various origin often stressed because having resisted to environmental conditions or hard physical and/or chemical treatments (disinfection agents, UV treatment).

WHICH METHOD FOR THE ENUMERATION OF *PSEUDOMONAS* SPP ?

In this date, any standardized method for the enumeration of *Pseudomonas* and related flora in milk and milk products exists. Methods used appear particularly varied as testify the table below ; it describes the means set up by the 25 French milk payment laboratories (results on telephone investigation).

Protocols quoted for the *Pseudomonas* spp enumeration

Medium used	Incubation	Analysis frequency	Number of Laboratories	Confirmation test
CFC Biokar/Oxoid	22°C / 72h 22°C / 48h 25°C / 24 à 48h 30°C / 24 à 48 h	Rare	1 6 1 2 either 40%	no NF Standard tests oxydase oxydase, métabolism O/F according Kligler
Nutrient agar + pénicillin G (100 u.i)	7°C / 10 days 6°C / 10 days	Rare	1 1 either 8%	no
Medium GSP (Merck)	22°C / 4 to 5 days	Rare 10/month	1 either 4%	Psychotrophic flora in parallel
PCA	4°C / 10 days	Rare	1 either 4%	no
No analysis *Pseudomonas*			11 either 44%	

Within the framework of the analytical methods harmonization, ISO proposed a horizontal method for *Pseudomonas* spp enumeration in foodstuffs animal food (ISO/ WD 13720). The announced objective is to count all psychrophilic *Pseudomonas* spp, pigmented or not, which plays an important role in the deterioration of food products. The methodological definition described in the draft standard is as follows :
Bacteria of *Pseudomonas* genus which form colonies on Cetrimide agar (10 µg/mL final) and Cephalosporine (50 µg/mL final) agar (CFC) after incubation at 25°C (in 48 hours) and which, moreover shows the following characteristics :
- positive reaction to oxydase test in 10 seconds
- absence of glucose fermentation (in 24 hours at 37°C)

Note : The recommended tests are carried out after insulation of the colonies on ordinary nutritive agar and incubation 24 hours at 25°C.
The suggested method is similar with the standard method NF V 04-504 recommended for the *Pseudomonas* spp enumeration in meats and meat products.

STUDIES REALISED

Within the request of the joint working group FIL France ALF/AFNOR, a preliminary study was initiated at the Laboratory of Food Characterisation (UR "Typicity of foodstuffs") in order to evaluate the relevance of the application of the method for the enumeration of *Pseudomonas* spp in dairy products.

This study consists of :

- a bibliographic study relative to "the evaluation of *Pseudomonas* enumeration methods and related in the dairy products"
- a technical study aiming at usually comparing the method recommended by the draft standard ISO WD 13720 with the other methods routinely used in research and testing laboratories.

1 – Bibliographical synthesis

The document draws up an inventory of the media and their conditions of use for the enumeration of *Pseudomonas* spp or related flora (psychrotrophic flora, lipolytic flora, proteolytic flora, flora producing fluorescent pigments). It is based on the analysis of 135 scientific articles published between 1970 and 2003, in relation to the quoted problem.

The document is split in 4 parts for the following topics :

- description of enumeration methods for *Pseudomonas* and related flora : composition of the culture media, incubation conditions, flora counted, advantages and disadvantages.
- the characteristics of connected flora that are the flora most often counted jointly with *Pseudomonas* and related in a non intentional way
- the description of research methods for Burkholderia cepacia and pseudomallei (ex *Pseudomonas*)
- a few data relating to *Pseudomonas* spp prevalence in milk, and dairy products, and to *Pseudomonas* spp-related product deteriorations.

This document points out the diversity of the approaches used. We most frequently meet enumeration in two stages, first consisting in psychrotrophic flora counting followed by the differentiation of *Pseudomonas* and connected flora on the basis of pigment production or enzymatic activities (lipolysis, proteolysis). In the case of enumeration in single stage, CFC, Cetrimide and GSP media are most frequently quoted. However, it should be mentioned that if works relating to milk are numerous, only few studies deal with the same issue in cheeses and dairy products.

2 – Technical study

On the basis of collected data in the former bibliographic study, a technical study was carried out by the Laboratory of Food Characterization of ENITA of Clermont-Ferrand (France) . It was monitored by a piloting group of the joint FIL-France (ALF) / AFNOR working committee Microbiology in Dairy Products.
This study consists of :
- a quantitative comparison of the *Pseudomonas* enumerations and related obtained according to 3 different methodologies (medium nature, incubation temperature and duration), the recommended conditions by the draft standard ISO WD 13720 being used as bases for comparison.
The analyses were carried on 60 samples from 29 cheeses or cheese products chosen at the market stage and representative of the diversity of French technologies (raw milk, thermized or pasteurized, of bovine, ovine or caprine origin ; soft, semi-hard and hard cheese, with smear coat or not, mould cheese, fresh cheese).

The description of more 310 isolates (coming from the enumeration carried out in the preceding stage) for various criteria :
- reaction to oxidase test,
- glucose fermentation at 25°C,
- macroscopic description of colonies,
- microscopic description,
- Gram colouring reaction,
- growth at 4°C, 41°C at 25°C on selective media,
- production of pigment on King A and King B agar,
- description of caseinolytic, lipolytic and esterasic activities.

In the objective to evaluate the relevance of the *Pseudomonas* spp characterization criteria as described in the draft standard ISO/WD 13720.

The results of this work will be presented in a forthcoming publication of the "Lettre de CECALAIT".

CECALAIT'S LIFE

Study on the enumeration of somatic cells in the goat's milk

On the request of ANICAP and CNIEL, CECALAIT is on to undertake a study on the methods of somatic cells enumeration in goat milk. It starts on April and comprises two parts :
- an experiment - on the reference method by visual counting (microscope) in order to define a specific alternative for goat milk, by applying the method used by FDA in USA (use of an other dye different from the standard one for cow milk).
- the setting up of calibration samples for automatic cells counters specific to goat' milk, for use within the framework of milk payment.

Time needed to achieve the study will be about one year.

NEW EU REGULATION

AFLATOXIN

OJEU 13.12.2003 - Commission regulation (EC) n° 2174/2003 of 12th December 2003 aflatoxins

ADDITIVES

OJEU n° L24 – Commission directive 2003/114/EC amending directive 95/2/EC on the foods additives different of dyes and sweetening substances

OJEU 20.12.2003 – Decree of 19th December 2003 amending decree of 2nd October 1997 relating to additives which can be used in the processing of products for human consumption

SWEETENING SUBSTANCE

OJEU n° L24 – Directive 2003/115/EC amending directive 94/35/EC on the sweetening substances intented to be used in the foodstuffs

SALMONELLA

OJEU n° L325 – Regulation (EC) 2160/2003 on the control of *Salmonella* and other specific zoonotic agents present in the food chain

CONTAMINANTS

OJEU n° L332 – Directive 2003/121/EC relating to the sampling modes and analysis methods for the official control of the maximum contents for certain contaminants in the foodstuffs

FORTHCOMING EVENTS

Events ordered according to chronology

SMALL RUMINANTS		
26 – 30 October 2004 Zaragoza, Espagne	IDF 4th International Symposium on the ewes and goats milk : utilisation, production, markets, prospects	Mail: www.fil-idf.org/sheepgoat2004
DAIRY SUMMIT		
22 – 26 November 2004 Melbourne, Australia	**IDF world dairy summit**	www.idfmelbourne2004.com
INDOGENOUS ENZYMES		
20 – 22 April 2005 Cork, Irland	**IDF conference on indegenous enzymes in milk**	www.ucc.ie/IDFenzymes

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