ENUMERATION OF ***PSEUDOMONAS*** SPP IN DAIRY PRODUCTS: THE DIFFICULTY IN CHOOSING AN APPROPRIATE METHOD

PART 2: EXPERIMENTAL STUDY

On the demand of a mixed working group composed of IDF - ALF France and AFNOR (microbiology of dairy products section), a study was engaged to evaluate the pertinence for the dairy industry, of a horizontal method (proposal ISO/WD 13720) for the enumeration of ***Pseudomonas*** spp in foodstuffs and animal feeding stuffs. This study was organised in 2 parts:

- Inventory of enumeration methods described in scientific literature and of methods set up by French milk payment laboratories,
- Quantitative and qualitative comparison of 2 enumeration protocols (including the method proposed in the draft standard ISO/WD 13720).

Part 1 was the subject of a previous article in CECALAIT's Newsletter n° 48.

CONTEXTE

Within the framework of the harmonisation of analytical methods, ISO proposed a horizontal method for enumeration of *Pseudomonas* spp in foodstuffs and animal feeding stuffs (ISO/WD 13720).

The announced objective was to enumerate all psychrophilic *Pseudomonas* spp, pigmented or not, that play an important role in product deterioration. The methodological definition described in the draft standard is as follows:

➢ Bacteria of the genus *Pseudomonas* that form colonies on Cetrimide (10 mg/l), Fucidin (10 mg/l) and Cephalosporin (50 mg/l) agar (CFC) after incubation at 25°C (for 48 hours) and that also present the following characteristics:

- positive reaction, within 10 seconds, to the oxidase test
- absence of fermentation of glucose (after 24 hour at 37°C)

Remark: the recommended tests are to be carried out after isolation on ordinary nutritive agar and incubation 24 hours at 25°C.

The method proposed is identical to that of the standard V 04-504 destined for the enumeration *Pseudomonas* in meat and meat-based products.

As developed in the precedent article on the subject, *Pseudomonas spp* isolated from dairy products and their environment distinguish themselves by their hyper-adapted character, and therefore, their phenotypic diversity, amongst others. Furthermore, *Pseudomonas* induce in dairy products extremely variable deterioration such as: soft curd, jellification, low yield and modified setting times, appearance of red-brown marks on the cheese surface, or intense fluorescent yellow colours associated with more or less important taste and texture defects (by proteolytic, peptidic, lipolytic or esterase activities), or strong smells (secondary metabolites). Finally, it is interesting to specify that numerous Gram negative bacteria, otherwise assimilated to the *Pseudomonas* genus and henceforth re-classed into other taxonomic groups, provoke similar deterioration.

Pseudomonas spp and related bacteria are therefore clearly distinct in quantity and quality within different food matrices. Concerning the dairy industry, numerous and complex interrogations relative to the methodology and the objectives of enumeration have appeared:

- Does the method for enumeration of *Pseudomonas* spp in dairy products, such as it is proposed, allow the enumeration of *Pseudomonas* spp in their totality (there currently exist over 140 species). If not, does it allow enumeration of *Pseudomonas* spp implicated in deterioration phenomena related to pigment production, enzymatic, lipolytic, esterase, caseolytic or other activities?
- Does the method proposed induce a bias in the enumeration results of "non *Pseudomonas* spp"?.
- Considering the evolution in the knowledge of the genus since 1998, publication date of the standard V 04-504, are the methods for characterisation of suspected isolates (oxidase test, fermentation of glucose) still pertinent for the characterisation of *Pseudomonas* spp?
- Finally, is the recommended method for enumeration of *Pseudomonas* spp in meat and meat products the best adapted for enumeration of *Pseudomonas* spp in dairy products?

OBJECTIVES OF THE STUDY

The objective of the study, was therefore to compare several methods (culture media, temperature, incubation time) for the enumeration of *Pseudomonas*

spp in representative samples of the main cheese categories. The comparison concerns the quantitative enumeration values as well as the characteristics of the isolates that developed on the media used.

MATERIAL AND METHODS

▸ The Cheeses

29 cheeses or cheese specialities were collected, at the commercialisation stage, at different sales outlets or in different forms (fresh packaged, self-service or loose). They are divided up according to the following categories:

	Cows' milk		Goats' milk		Sheep's milk	
	Raw milk	Heated / Pasteurised milk	Raw milk	Heated / Pasteurised milk	Raw milk	Heated / Pasteurised milk
Soft cheese with surface mould	N=2	N=1	N=2	N=1	N=1	N=1
Soft cheese washed / smeared	N=2	N=1				
Semi-hard cheese	N=2	N=1	N=1	N=1	N=1	N=1
Hard cheese	N=1	N=1				
Veined cheese	N=1	N=1			N=1	
Fresh cheese		N=5				N=1

For each cheese, and where possible, the analyses were carried out on samples taken from the centre and the rind of the cheese. In certain cases, several batches of the same type of cheese were analysed. In total, 57 samples were included in the study.

1° Preliminary step to choose incubation conditions and enumeration media

▸ Objectives

This preliminary stage was carried out in order to simply compare the results of the enumeration of *Pseudomonas* spp and related species in 2 cheese samples (Cheese n° 1 et Cheese n° 2). The comparison was carried out using several parameters: incubation conditions (time/temperature couple) and media used (media and conditions were chosen from the bibliographic data obtained previously).

▸ Samples

Cheese n° 1 is a semi-hard cheese made from cows' milk. It is salted on the surface then washed several times during the 4 weeks of maturation. The surface flora is abundant and diverse.

Cheese n° 2 is a goats' milk cheese with lactic fermentation, for which the maturation is 6 days. The fungal surface flora is abundant but less diverse than that of Cheese n° 1.

These 2 cheeses are made with raw milk and adjunction of selected starters.

▸ Preparation of initial suspensions, decimal dilutions and inoculation

For each cheese, 25g of sample (rind and centre) were taken in sterile conditions and put in a blender bag. 225 ml of buffered peptone water was added for a final concentration of 1/10th. After blending for 1 min., decimal dilutions of the initial suspension were obtained using buffered peptone water. Inoculations on the different solid culture media were carried out using the « Spiral » inoculator (AES) according to the standard method V 08-100. For each dilution, inoculation was carried out in triplicate.

▸ Culture media and incubation

6 culture media, elaborated with basic CFC agar supplemented with different anti-microbial agents and selected on the basis of the bibliographic data, were tested:

- CFC agar without addition of anti-microbial agents.
- CFC agar supplemented with Irgasan (25 mg/l).
- CFC agar supplemented with Nitrofurantoin (350 mg/l).
- CFC agar supplemented with Cetrimide (10 mg/l) and Nalidixic acid (10 mg/l).
- CFC agar supplemented with a mixture of Cetrimide (10 mg/l), Fucidin (10 mg/l) and Cephaloridin (50 mg/l), or CFC. This medium is recommended by the draft standard ISO/WD 13720.
- CFC agar supplemented with Penicillin G (100 000 UI) and Pimaricin (10 mg/l), or GSP.

All the Petri dishes were inoculated in aerobic conditions.

4 incubation temperatures were compared: 4°C, 6°C, 17°C and 25°C.

▸ Enumeration

Observation and counting of the Petri dishes were carried out after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11

days of incubation. The enumeration was carried out and the results expressed according to the recommendations of the manufacturer of the Spiral apparatus and in accordance with the standard V 08-100. Supplementary observations, concerning the morphology and differentiation of colonies, pigment production, growth of yeast and moulds, were noted.

RESULTS

At 4°C, whichever medium was used, colonies could be detected on the surface of the Petri dishes after 7 days of incubation. However, the appearance of the colonies was difficult to describe and no pigment production was observed, even after 10 days of incubation.

At 6°C, the results were very similar to those observed at 4°C, apart from the fact that pigmentation was observed for certain colonies after 8 days of incubation.

At 17°C, differences were observed between the media tested. The medium supplemented with nitrofurantoin does not allow easily detectable fluorescent pigmentation, even after 4 days of incubation. Also, after 3 days, mould develops abundantly. However, the other media allow differentiation of pigmented colonies after 48 hours of incubation, which appears very clearly after 4 days.

At 25°C, yeast and mould develop abundantly, on CFC agar, CFC agar with cetrimide and Nalidixic acid, CFC agar with nitrofurantoin and CFC agar with Irgasan, after 24 hours of incubation. On the CFC agar with CFC, growth of yeast and mould appears after 48 hours, whilst the CFC agar with GSP seems to totally inhibit the growth of fungal flora.

Concerning the differentiation of colonies on the basis of their pigmentation: apart from the medium supplemented with nitrofurantoin which does not allow observation of fluorescence, all the other media allow clear distinction of pigmented colonies after 48 hours.

▸ Quantitative criteria

For the 2 types of cheese analysed, the enumeration values (uncorrected) varied between 1.9E+03 and 5.5E+04 (Cheese n° 1) and the inferior detection limit and 2.1E+02 (Cheese n° 2)

CFC agar without addition of anti-microbial agents: This medium serves as a reference for comparison with the other media. Without addition of selectivity agents, only the temperature and aerobic incubation conditions will have an effect on the selection of the flora counted.

CFC agar supplemented with Irgasan (25 mg/l): This medium allows rapid growth of yeast and mould, and moreover, does not allow discrimination of pigmented colonies. Also, the recovery rate appears to be clearly inferior to those observed with the other media tested.

CFC agar supplemented with Nitrofurantoin (350 mg/l): This medium allows rapid growth of yeast and mould, and moreover, does not allow discrimination of pigmented colonies. Also, the recovery rate appears to be clearly inferior to those observed with the other media tested.

CFC agar supplemented with Cetrimide (10 mg/l) and Nalidixic acid (10 mg/l): Although this medium allows the differentiation of pigmented colonies, it allows recovery of the sought-after flora at much lower levels than the other media. This difference is particularly important for Cheese n° 2 cheese, which seems to present a low concentration of *Pseudomonas* spp and a high concentration of fungal flora.

CFC agar supplemented with a mixture of Cetrimide (10 mg/l), Fucidin (10 mg/l) and Cephaloridin (50 mg/l) : This medium is recommended by the draft standard ISO/WD 13720. It presents interesting qualitative characteristics, an easy revelation of pigment producing colonies, a good inhibition of fungal flora at 17 and 25°C and a good development of colonies for macroscopic description (notably mucoid colonies). The potential of this medium should be compared with that of CFC agar with GSP.

CFC agar supplemented with GSP: This medium presents similar characteristics to those evoked above. However it presents the following additional advantages: growth of fungal flora is slowed or even inhibited at all the temperatures tested; The rate of recovery is superior, notable as far as Cheese n° 2 cheese is concerned. Nevertheless, it is important to note that in this last case, colony development is slower than on the previous medium.

CONCLUSION

For the 2 types of cheese analysed, the CFC agar with GSP offers a higher rate of recovery of suspected *Pseudomonas* spp than the other media tested. None of the other media can guarantee an absence of growth of non *Pseudomonas* spp. However, the CFC agar with GSP seems the most efficient in limiting the development of fungal flora.

Concerning incubation temperatures, it clearly appears that 4°C and 6°C allow easy reading of results only after 10 or even 12 days, which seems incompatible with auto-control constraints of the food industry.

Temperatures of 17°C and 25°C seem favourable for the sought-after flora. They lead to identical enumeration values after 72 and 48 hours of incubation, respectively.

In the next phase of the study, the CFC agar with GSP was tested for comparison with the medium recommended in the draft standard ISO/WD 13720 and with CFC agar (without addition of anti-microbial agents). Incubation was at 17°C for 3 days in order to facilitate macroscopic observation of colonies.

2° Comparison of CFC agar with GSP and CFC agar with CFC (draft standard ISO/WD 13720) for enumeration of ***Pseudomonas*** spp in different cheeses and cheese specialities.

▸ Samples and sample preparation

Samples were prepared and inoculated according to the protocol described previously. Counting was carried out after 3 days of incubation at 17°C.

▸ Enumeration

For 14 of the 57 samples analysed, the CFC agar with GSP allowed a greater recovery rate of suspected *Pseudomonas* than the medium proposed by the draft standard ISO/WD 13720. The differences varied from 1 to 5.5 log.

For 19 of the 57 samples, the differences in enumeration values were minimal, as they were absent or inferior to 1 log.

For 25 of the 57 samples, the enumeration values were below the detection limit, whichever method was used.

Finally, the medium recommended by the draft standard ISO/WD 13720 allowed a higher recovery rate in only one case, a veined cheese made from raw cows' milk.

▸ CONCLUSION

The CFC agar with GSP seems to allow a better recovery of suspected *Pseudomonas* than the CFC agar with CFC recommended by the standard ISO/WD 13720. However, at this stage in the study, nothing permits one to know if the additional flora is constituted of *Pseudomonas* spp or other microbial flora. It is necessary to note that under the conditions used in the study, no mould was detected on the CFC agar with GSP.

▸ Characterisation of the isolates

Whenever possible, the square root of the total number of isolates was randomly harvested from the CFC agar with GSP. The colonies were purified, then characterised according to the criteria described in the draft standard ISO/WD 13720 and according to additional criteria, notably their suspected deteriorating metabolic activities.

▸ Reactives and methods: Characterisation criteria of the draft standard ISO/WD 13720

Oxidase test reaction: the reactive is 1g/l of N,N,N',N-tetramethyl-p-phenylenediamine dichloride in water deposited on filter paper. Concerning this test, and contrary to the operating conditions described in the draft standard ISO/WD 13720, the apparition of a violet coloration was considered as being significant of a positive test result after a response time of up to 1 min. In effect, amongst the isolates studied important variability was noted on the apparition time of the violet coloration.

Fermentation of glucose at 25°C: Composition in g/l: Enzymatic digest of casein (10), yeast extract (1,5), sodium chloride (5), glucose (10), bromocresol purple (0,015), agar (15). Contrary to the protocol described in the draft standard ISO/WD 13720, incubation was carried out at 25°C and not at 37°C, which seems more coherent for the characterisation of psychrotrophic micro-organisms.

▸ Other criteria:

- Gram coloration reaction (differentiation Gram + / Gram -)
- Microscopic observation of micro-organisms (morphological description) after coloration
- Growth at 25°C on CFC agar with CFC medium after subculture of a colony with a toothpick
- Pigment production on King A agar
- Pigment production on King B agar
- Growth at 4°C for 7 days on CFC agar with CFC
- Growth at 41°C for 2 days on CFC agar with CFC
- Lipolytic activity on trybutyrin agar (incubation at 25°C for 48 hours)
- Esterase activity on egg agar (incubation at 25°C for 48 hours)
- Caseolytic activity on milk agar (incubation at 25°C for 48 hours)

In total, 465 isolates were characterised, of which 339 with all the tests cited above.

▸ Pertinence of the media

67% of the isolates counted on CFC agar with GSP were positive for the oxidase test and did not ferment glucose. These isolates would therefore be considered as *Pseudomonas* spp according to the criteria described in the draft standard ISO/WD 13720.

77% of the isolates collected from CFC agar with CFC (after isolation on CFC agar with GSP and subculture with a toothpick and incubation at 25°C) would be considered as being *Pseudomonas* spp according to the criteria described in the draft standard ISO/WD 13720.

Taking into account the methodological bias linked to the non-discrimination of eventual duplicates, this difference does not appear to be significant.

▸ Other data

25% of isolates from CFC agar with GSP identified as being *Pseudomonas* spp according to the criteria described in the draft standard ISO/WD 13720 were unable to grow on CFC agar with CFC.

This data confirms the greater pertinence of CFC agar with GSP for enumeration of Pseudomonas *spp in dairy products.*

▸ Pertinence of characterisation criteria

93,5 % of the micro-organisms isolated on CFC agar with GSP that were positive for the oxidase test and that did not ferment glucose, are Gram negative bacillus.

This result shows that Gram coloration and microscopic observation do not bring any supplementary elements for identification of *Pseudomonas* spp isolated from the selective media used (for use in auto-control analysis in the food industry). This result is in accordance with bibliographic data and previous analyses that show that the majority of non *Pseudomonas* flora isolated from the media tested are Gram negative bacillus. This remark is true if it is accepted that mould yeast can hinder reading of the Petri dishes and false the enumeration values by competitive inhibition of *Pseudomonas* spp flora.

19 % of the micro-organisms isolated from CFC agar with GSP that did not ferment glucose were negative for the oxidase test.

This result suggests that the oxidase test is more pertinent for better differentiation of non Pseudomonas *spp flora susceptible to develop on the media tested.*

Additional Characteristics of the isolates

Activity	All isolates (1) n= 465	Suspected *Pseudomonas spp* isolates (2) 166 < n < 238
Pigment Production on King A agar	2 %	3 %
Pigment Production on King B agar	24 %	37 %
Growth at 4°C	34 %	43 %
Growth at 41°C :	52 %	36 %
Lipolytic activity	42 %	42 %
Esterase activity	44,5 %	57 %
Caseolytic activity	36,6 %	34 %

(1) Percentages obtained for all the isolates from CFC agar with GSP.

(2) Percentages obtained for all the isolates from CFC agar with GSP, identified as *Pseudomonas spp* according to the criteria: Gram negative bacillus, that are positive for oxidase and that do not ferment glucose.

The characteristics studied, even though they are not particularly interesting for the analysis of the deterioration power of the sought-after flora, do not constitute criteria for the identification of *Pseudomonas* spp.

To answer our initial interrogations

- *Does the method for enumeration of* Pseudomonas *spp in dairy products, such as it is proposed, allow the enumeration of* Pseudomonas *spp in their totality (there currently exist over 140 species). If not, does it allow enumeration of* Pseudomonas *spp implicated in deterioration phenomena relative to pigment production, enzymatic, lipolytic, esterase, caseolytic or other activities?*

Considering the information collected from the review of the bibliographic and from the experimentation, it appears that the proposed method does not allow enumeration of *Pseudomonas* spp, or the enumeration of those *Pseudomonas* spp implicated in the deterioration of dairy products. However, it seems utopian to think that such a classic microbiological method exists. It would, however, be interesting to propose an enumeration method to which changes could be brought in order to bring to the fore additional characteristics, such as the production of proteolytic or lipolytic activities for example. Further experimentation needs to be carried out in this direction.

- *Does the enumeration medium (CFC agar with GSP) presented here as an alternative to that*

proposed in the draft standard ISO/WD 13720, induce a bias in the enumeration results of "non Pseudomonas *spp flora"?.*

The answer is yes. But, on this point no better method was found, neither in the bibliography nor during the experimentation.

- *Are the methods of characterisation of the suspected isolates (oxidase test, fermentation of glucose) still pertinent considering the evolution in the knowledge of the genus since 1998, publication date of the French standard V 04-504 ?*

Considering the evolution of taxonomic data relative to the genus *Pseudomonas*, it appears clearly that the recommended tests are not pertinent enough to discriminate *Pseudomonas* spp from other flora regularly isolated on the media tested. However, no additional pertinent and easy-to-use tests were brought to the fore for use in a "classical" microbiological analysis laboratory.

CFC agar with GSP allows a better recovery of ***Pseudomonas*** spp and related species from the cheeses tested. It limits the growth of fungal flora, and allows the production of pigment to be brought to the fore, and does not lead to a higher enumeration of non ***Pseudomonas*** flora (in comparison with CFC agar with CFC).
The characterisation criteria of the counted flora (for a corrected expression of results) proposed by the standard ISO/WD 13720 are not sufficiently pertinent, but no other could be proposed for routine analyses in the laboratory.

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The results of this study were presented in Parma, Italy, in April 2004, where the IDF analytical week (dairy products) and the ISO/TC 34 SC9 meeting (all products) were being held jointly.

For the continuity of the study, during the IDF meeting it was decided to launch a comparative study between CFC agar, GSP agar and the new medium: CFC agar with GSP, for a more widespread testing of this new medium in different laboratories and in different dairy product matrices. The same protocol as that presented at the ISO SC9 meeting was proposed to test all food products. The draft ISO/WD 13720 describing the horizontal method, it was desirable to compare the 3 media on other matrices.

This study is co-ordinated by CECALAIT. The protocol was diffused in September for a return of results for 20th February 2005 at the latest. Each participating laboratory must carry out the comparison on their own samples.

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