COMPARISON OF MEDIA FOR THE ENUMERATION OF *PSEUDOMONAS*

DETERMINATION OF INCLUSIVITY AND EXCLUSIVITY (ACCORDING TO ISO 16140 STANDARD)

In the absence of a standard for the enumeration of *Pseudomonas* in milk and dairy products, the mixed standardisation work group IDF-ALF France and AFNOR V08B (microbiology of dairy products section), proposed a new study to the IDF in 2002 for the standardisation of this method. This new study was submitted to a vote and approved by the member countries (16 positive votes out of 21 voters).
A horizontal draft standard for the enumeration of *Pseudomonas* (ISO/WD 13720) exists, but the CFC medium described in this standard and usually used for meat, does not seem suited for analysis of milk and dairy products. An enquiry was realised at the IDF level, which showed effectively that even if the CFC medium was the most used, no data concerning its adaptability to milk and dairy products exists.

The work group entrusted the development of a new medium to Françoise LERICHE (ENITA Clermont-Ferrand), *Pseudomonas* specialist (see articles published in CECALAIT's Newsletter, n° 48 and n° 51). On top of this study, a new medium, PPA (Penicillin Pimaricin Agar), was proposed.
In 2004, these results were presented to the IDF JAT group, which decided to carry out a comparison study of 3 media: CFC agar, GSP agar and PPA. These tests were led jointly by IDF («microbiological harmonisation») and ISO (TC 34 / SC 9), to study the possibility of using one medium for all food products.
- Concerning dairy products, the results obtained in this study are, on average, higher for PPA, quite close for PPA and GSP agar, and a lot lower for CFC agar.
- For other products, the results obtained in this study are equivalent for the 3 agars.
These results were presented during the ISO SC9 meeting in June 2005 and two sectorial standards were proposed: one for milk and dairy products, and the other for meat and meat food products (ISO 13720).

However, to finalise a method for dairy products, complementary tests seemed necessary. That is why the specificity and the selectivity of the enumeration media were tested by studying the confirmation tests in parallel.

PRINCIPLE:

Firstly, the study consisted in comparing the growth of target and non-target strains on 4 different agars, of which one non-selective was used as a reference, and in calculating the productivity of the media. The confirmation tests were implemented, which permitted the evaluation of the totality of the method. Moreover, the study of the target and non-target reference strains has allowed the strains to be used in the media performance tests, which must now figure in any new ISO standard, to be selected.

1 - MATERIAL AND METHODS

1.1 - PROTOCOL

The protocol described in the ISO 16140 standard was followed.

1.1.1 - ENUMERATION:

- Culture of strains in a non-selective broth (Brain Heart Infusion) incubated approximately 18 hours at 25°C.
- Dilution of this culture to obtain 50 to 150 colonies per dish.
- Inoculation of 2 dishes per medium, using the Spiral system, on 4 different media:

- CFC agar (AES): described in the ISO 13720 standard, incubated 48 hours at 25°C;
- GSP agar (MERCK): supplemented with penicillin at 100 000 UI/l (Calbiochem) and pimaricin at 0.01g/l (Molekula), incubated 72 hours at 25°C;
- PPA: made up with the CFC base and the GSP agar supplements (penicillin and piramicin), incubated 48 hours at 25°C;
- TSA (AES): Trypticase Soja Agar, incubated 48 to 72 hours at 25°C, reference medium to verify strain growth and to calculate the productivity of the selective media for a same time of incubation (according to ISO 11133-2 standard):

$$\text{PR (productivity)} = \frac{\text{Number of colonies on the medium being tested}}{\text{Number of colonies on TSA}}$$

1.1.2 – CONFIRMATION:

- , The confirmations were realised from a colony isolated from each selective agar, that is to say 3 colonies per strain.
- The isolations of these colonies were realised on nutritive agar incubated 24 to 48 hours at 25°C, before the realisation of both confirmation tests:

- **Detection of oxidase**: the method described in the ISO/WD 13720 standard and 3 different commercial tests were compared, noting, for each result, the time of appearance of the violet colour.
- **Glucose fermentation**: glucose agar (Biokar Diagnostics) and Kligler Hajna agar (bioMérieux), media recommended in the ISO/WD 13720 and NF V04-504 standards, were tested in parallel. To study the influence of incubation time, the reading was

realised after 21, 24 and 27 h at 25°C. A second comparison was then realised between glucose agar and the O/F medium (bioMérieux), but only on one colony per strain. The O/F medium is effectively recommended in the revision of the ISO 21528 standard, parts 1 and 2, concerning *Enterobacteriaceae*, and therefore chosen instead of glucose agar in the revision of the ISO 13720 standard.

• When the species was badly defined or in the case of doubt, one API 20NE or API 20E (bioMérieux) gallery, according to the case, was realised. For *Pseudomonas*, when the API 20 NE gallery did not identify the species, a complementary identification was realised in BioMérieux's laboratories: from additional biochemical tests to RNA 16s sequencing, according to the difficulty of the identification. 5 strains were tested like that, and the species of only one strain was not precisely determined.

2 – TESTED STRAINS:

62 different target and non-target strains were tested:

- 49 from 6 French laboratories: 31 from milk and dairy product samples, of which mainly cheeses, 13 from environmental samples from dairy industries, 2 of undefined dairy origin and 3 of food origin ,
- 2 commercial mould strains,
- 10 reference strains,
- 1 strain of undefined origin.

Of which 48 strains of dairy origin, this allowed the inclusivity and exclusivity results to be obtained:

- 24 *Pseudomonas* target strains: 13 *P. fluorescens*, 3 *P stuzeri*, 2 *P. aeruginosa*, 2 *P. aureofaciens*, 2 *P. fragi*, 1 *P. pseudomallei* and 1 *P. spp*.

- 24 non-target strains that are known to interfere with *Pseudomonas* strains in dairy products: 10 *Aeromonas*, 5 *Enterobacteriaceae*, 6 Gram negative and glucose fermentation negative strains, 2 moulds and one yeast.

3 - RESULTS

Only results obtained on the strains of dairy origin were considered: various dairy product samples, mainly cheese, environmental samples during the process of these products and moulds used for cheese ripening.

3.1 – INCLUSIVITY

The inclusivity (or specificity) is the capacity of the method to detect target strains. The presence of these strains is therefore tested on the selective media studied.

Table 1: results of the inclusivity study expressed in percentage of detected strains

n = 24	Before confirmation			After confirmation		
Media	CFC	GSP	PPA	CFC	GSP	PPA
PSEUDOMONAS	100%	96%*	96%*	100%	96%*	96%*
* Strain B'1 (*P. stutzeri*): PR= 3.3 on CFC and PR = 0 on GSP and PPA						

All the *Pseudomonas* strains grew on all the media (PR of 0.6 to 4.1) except an undetected *P. stuzeri* strain on PPA and GSP media, which leads to a weaker specificity for both these media in comparison with CFC.

Results obtained before and after confirmation were identical, as all the *Pseudomonas* strains proved to be positive for oxidase and negative for glucose fermentation.
The results obtained on the reference strains show that 3 out of 8 *Pseudomonas* strains were identified on CFC medium, but few or none on GSP and PPA media.

These results were not taken into account because the reference strains did not come from dairy products and the genus *Pseudomonas* does not have stable physiological characteristics during preservation, particularly during the freezing.
Nevertheless, they permitted the strain *P. fluorescens* ATCC 13525 to be selected for the PPA medium performance tests.

3.2 - EXCLUSIVITY

The exclusivity (or selectivity) is the absence of interference of non-target strains. It is tested by the non-detection of non-target strains on the selective media studied.

Table 2: results of the exclusivity study expressed in percentage of non-detected strains

n = 24	Before confirmation			After confirmation		
Media	CFC	GSP	PPA	CFC	GSP	PPA
Gram- glucose- n=6	0%	33%	50%	50%*	67%*	67%*
Aeromonas n= 10	40%	0%	0%	100%	100%	100%
Enterobacteriaceae n=5	60%	40%	40%	100%	100%	100%
Yeasts and moulds n=3	0%	100%	100%	100%	100%	100%
ALL THE STRAINS	29%	29%	33%	88%	92%	92%

*3 strains confirmed as *Pseudomonas*: *Comamonas acidovarans* and *rkholderia cepacia* detected on all the media and *Shewanella putrefaciens* only detected on CFC.

- Before confirmation:

The 3 yeast and mould strains were detected on CFC medium but did not develop on PPA and GSP media, which confirms the use of piramicin, an antifungus, in both these media.

Likewise, the 6 Gram negative and glucose fermentation negative strains developed on CFC agar, but 2 of these strains did not grow on GSP medium and 3 on PPA medium.

The 10 *Aeromonas* strains grew on GSP and PPA media, but 4 did not develop on CFC medium.

Concerning the 5 enterobacteria strains, 3 did not grow on CFC medium and 2 on GSP and PPA media.

Globally, the results were better on PPA medium than on CFC and GSP media.

- After confirmation:

Nearly all the strains were confirmed as not belonging to the genus *Pseudomonas*. The specificity is therefore 100 % for all strain categories, except for 3 Gram negative and glucose fermentation positive strains.

Globally, the results are a little better on PPA and GSP medium than on CFC medium.

The results obtained with the reference strains, not noted in the table above, allowed the strains *Escherichia coli* ATCC 25922 and *Staphyloccocus aureus* ATCC 25923 to be selected for the performance tests on PPA medium, indeed none of the 3 tested media detect these strains.

3.3 - CONFIRMATIONS

The previous exclusivity results show the necessity of the confirmation step, which concerned the detection of oxidase and glucose fermentation. Moreover, this step seems to be quite important as all the *Pseudomonas* strains were identified and only 3 non-target strains out of 24 were not eliminated during this step.

A – Oxidase detection:

Generally, even if the oxidase test appears to be simple, it includes many difficulties linked to different factors:

- Disc impregnation: For the tests with a paper disc moistened with reagent (reference method and test « B »), the quantity of reagent must be neither too little nor too much, or there is a risk that the violet colour does not appear.
- Colour and texture of colonies: In this study, a red pigmented *Serratia* strain could not be tested. Likewise, it is difficult to deposit very dry colonies, such as mould and yeast strains, on the test support. In this case, the aspect of the colonies is sufficient to withdraw them from confirmation.
- Maximum time of appearance of the violet colour: It must be longer than the recommended time in the test instructions or in the standardised procedure. Actually, for the reference method, this limit is 10 s. For 4 *Pseudomonas* strains a longer time, about 15 to 20 s instead of 10 s, was observed. A time limit of 30 s will be recommended in the draft standard. In the case of test « C », the time limit of 5 s seemed to be a little too short.
- Interpretation of the colour: As with any visual reading, it is sometimes difficult to appreciate the colour intensity.

In view of all the results, the comparison of the reference method and the 3 commercial tests showed equivalent results: on the 62 strains tested, only 3 gave divergent results. These results are noted in the table above. The influence of the incubation time on nutritive agar and of its cold storage time was tested. According to the results, these factors scarcely influence the oxidase test. However, an incubation of 24 hours on nutritive agar seems to be preferable for the reference method, in particular for the strain *Stenotrophomonas maltophilia*, normally negative for this test.

Table 3: Influence of incubation time on nutritive agar incubated 24 to 48 h at 25°C and its storage 5 days at 4°C on the oxidase test result

Strain	Identification	A			B			C			D		
		24h	48h	4°C	24h	48h	4°C	24h	48h	4°C	24h	48h	4°C
HA: negative sample	*Hafnia alvei*	-	-	-	-	-	-	-	-	-	-	-	-
F5: positive sample	*P. fluorescens*	+	+	+	+	+	+	+	+	+	+	+	+
1	*Stenotrophomonas maltophilia*	-*45s	+ 20s	+ 20s	-	-	-	-	-	-	-	-*30s	-*30s
06-183-01	*P. putida*	+	+	+	+	+	+	+	+	+	+	+	+
MSII 2.1	*P. fluorescens*	+	+	+*	+	+	+	-	(+)	+	+	+	+
MS 4.1.3.9	*P. fragi*	+	+	+	+	+	+	+	+	+	+	+	+
ATCC 12633	*P. putida*	+	+	+	+	+	+	(+)	+	+	+	+	+
ATCC 13525	*P. fluorescens*	+	+	+	+	+	+	+	+	+	+	+	+
CIP 103022	*P. stuzeri*	+	+	+	+	+	+	+	+	+	+	+	+

Methods	Oxidase +
A: reference	< 30s
B:	10 à 30 s
C:	< 5s
D:	20 à 60 s

Oxidase -*: very light colour
Oxidase +*: light colour
Oxidase (+): For C > 5 s

B – Glucose fermentation:

On all 62 strains, and on 3 isolates per strain, the results obtained with glucose agar and Kligler medium for the different incubation times (21, 24 or 27 hours at 25°C) were the same.
The results are therefore identical concerning the comparison of glucose and O/F media, which was realised later on 1 isolate per strain and without oil adjunction on the surface for both media. However, it is more difficult to interpret the negative results on O/F medium: the colour of the tube stays green or the top half is yellow and the bottom half is green. As glucose agar is in a taller and thinner tube, the colour change appeared only on about 1 cm of the surface of the tube. To facilitate reading, it was perhaps necessary to add oil to the surface of the O/F tube to create anaerobic conditions, but that means extra manipulations.

CONCLUSION: PROPOSITIONS FOR THE ELABORATION OF THE STANDARD ON MILK AND DAIRY PRODUCTS

1. Enumeration medium:

The results of the inclusivity study are slightly in favour of the CFC medium; only one strain out of 24 was different. On the other hand, the exclusivity results show a slightly better performance for PPA medium.

The results obtained by F. LERICHE on milk and dairy samples (CECALAIT's Newsletter n° 48 and n° 51) and in the ISO/IDF study, show that the enumeration of *Pseudomonas* obtained on PPA medium is much higher than on CFC medium.
PPA and GSP media produce similar results, slightly more favourable for PPA. However, because of the colour of GSP it is difficult to distinguish the aspect of the colonies, in particular their pigmentation, and its agar-rich base does not favour the culture of *Pseudomonas* .

It is therefore recommended, in the draft standard, to use PPA medium incubated 48 +/- 2 h at 25°C.

2. Confirmation:

This study showed the necessity of confirmation tests and that oxidase and glucose fermentation tests were adapted to this method.
During the ISO SC9 meeting in April 2007, for the revision of the ISO 13720 standard concerning meat and meat products, the possibility of keeping only the oxidation test for the confirmation was proposed, the glucose fermentation then becoming optional.
This proposition, studied during the IDF analytical week in May 2007, was refused by the «microbiological harmonisation» group. Indeed, it seemed very dangerous to propose a two-tier reference method, which could end in the presumption of *Pseudomonas*.

Even if the 3 glucose fermentation tests studied gave equivalent results, glucose agar seems more suitable:

- It is easier to prepare than Kligler agar, for which the agar must be solidified on a slope after regeneration,
- It is not necessary to add oil to the surface,
- It is easier to read than O/F medium.

It is therefore recommended for this standard the use of oxidase confirmation tests, with a reading time of less than 30 s and glucose fermentation on glucose agar incubated 24 +/- 3 h at 25°C.

3. Other propositions:

- During the last IDF meeting, it was decided to publish this standard as a **technical specification** in order to shorten the publication time, and to test this new method before its real standardisation.
- In the scope of application, the **possibility of analysing environmental samples** of dairy products was added.
- Following the results of this study, t**he media performance tests** were added in part.
- Important details concerning the **preparation of the pimaricine solution** and the storage conditions of the solutions and media were added.

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