REVIEW OF THE ACTIVITY OF THE « TBX MEDIUM » WORK GROUP OF THE AFNOR COMMISSION V08B « FOOD MICROBIOLOGY »

PART 2 : WORK GROUP ACTIVITIES

Tryptone-bile-glucuronide (TBX) and pepton-tergitol-glucuronide (PTX) agars are two chromogenous media, which allow the enumeration of *E. coli* by their positive character for β-D-glucuronidase, present in about 95% of *E. coli* found in food products. *E. coli* 0157:H7 for example, is deprived of this character.
Since 1993, the medium PTX is described in a routine standard at a national level (NF V 08-053). However, following the non-commercialisation of tergitol 7, a selective agent in this medium, it was replaced, at the end of 2002, by the medium TBX. This medium is standardised at an international level since 2001 (ISO 16649 parts 1 and 2). TBX had been selected following the ISO SC9 meeting in 1997, where comparisons had been presented on various matrices, with various methods and chromogenous media. However, taking into account these results, TBX did not seem to possess much higher performances than the other chromogenous media.

With the use of this new media, some laboratories met difficulties, particularly for the analysis of dairy products. These observations were described in a first article in "La Lettre de CECALAIT" n° 54. Following these reports, the AFNOR Commission V08B decided to create a work group, which must gather and study these observations and try to provide solutions. Organised by AFNOR, and animated by P. ROLLIER of CECALAIT, this work group is composed of about 10 suppliers and users. The group has met 3 times between 2004 and 2005. The activities and conclusions of this work group are presented in this second article.

1) COMPARISON STUDY OF THE METHODS LED BY THE AFSSA

Following remarks from the European Milk network, the AFSSA led a comparison study of different methods. Part of this study was presented to the TBX work group. The conclusions concerning the TBX medium are described below:

- The study dealt with the comparison of different parts of the IDF standard 16649:2001 (part 1: inoculation on membranes and on the surface ; part 2: inoculation in the mass ; part 3: MPN method). These tests were carried out with TBX media provided from only one supplier.
- Concerning the use of TBX (parts 1 and 2), on naturally contaminated cheeses, the results showed that part 1 is either equivalent to part 2, or allows recovery of more *E. coli*, particularly for samples containing stressed bacteria. However, part 1 requires an additional step of recuperation on a membrane, which is not convenient to set up. That is why almost no laboratories apply this method.

Then, the studies presented in paragraphs 2 and 3 have been realised following the protocol of part 2 or the quasi equivalent standard NF V 08-053:2002.

2) PROBLEMS POSED BY THE READY-TO-USE MEDIA

The work group has been particularly interested in the media to liquefied, which seem to pose more problems than the media reconstituted from powder. Indeed, some laboratories noticed that the ready-to-use TBX media in flasks seems to be more fragile and heat sensitive than the dehydrated media, with, in particular a more diffuse colony colour .

Then, the laboratory L2 compared, on naturally contaminated raw milk cheeses and on one of CECALAIT's proficiency tests in milk, ready-to-use or dehydrated TBX media from one supplier:

- In the raw milk cheese samples, this laboratory observed about the same or slightly lower numbers than with the dehydrated medium. Only one sample shows a very important underestimation (1.7 log).
- In the proficiency test samples, the differences are higher (between 0.6 and 0.8 log). They can be explained by the nature of the samples: they are artificially contaminated with a mix of 3 strains and contain a bacteriostatic preservative which can cause additional stress to the bacteria.

Another laboratory noticed that a medium prepared with a non pressure-sealed dehydrated medium is less effective than after pressure-sealing, but this practice is not advised by the suppliers.

CECALAIT also observed a decreasing performance in time on a dehydrated medium (see performance tests described below).

A compound contained in the medium TBX is probably sensitive to various factors, particularly to heat. It is perhaps necessary to sensitise the laboratories on the good practices of use of the ready-to-use media: for example, the storage temperature which can act on the stability of the chromogen, the limit of its heating time and its rapid use.

3) MEDIA PERFORMANCE TESTS

The work group therefore studied the performance tests to see if they were often implemented by the laboratories and if they could underline a defect of growth.

3.1 General principles of the performance tests

The protocol for the realisation of these performance tests and the strains to use are described generally in the ISO/TS 11133-2 standard. They must be realised by the laboratories which use dehydrated media. For the medium TBX, the strains to test are described in the routine standard NF V 08-053. If the media sterility and growth tests are successful, they are validated. For each growth test, the medium to evaluate is compared to a non-selective reference medium, generally TSA (Trypton-Soja-Agar).
3 growth tests are described:

- **Productivity**: concerns strains numbered or sought after by the tested medium and permits appreciation of the performance of the medium for the growth of these strains.
- **Selectivity**: on the contrary, concerns strains that are not numbered or sought after by the tested medium. The selectivity measures the capacity of the medium to totally inhibit these strains.
- **Specificity:** in the same category, allows evaluation of the culture of non characteristic strains.

Concerning the medium TBX, the protocol and the strains described in the standard to test the selectivity, seem to be suitable. However, there was a problem concerning the reference of a strain recommended for specificity in the NF V 08-053 standard. The work group studied this point and 2 strains have been proposed, as substitutes, to the Commission V 08B on 22nd March 2005 (point 4 – PV 165).
In the study described below, the work group particularly analysed the productivity tests in the user laboratories.

3.2 Productivity tests on TBX medium

3.2.1 Productivity tests realised by an analytical laboratory at the beginning of 2003:

The productivity tests were realised on a strain of *E. coli*, isolated from food products and inoculated in the mass with the media PTX and TBX, different from the strains described in the NF V 08-053 standard. These tests underlined the inhibition of *E. coli* on all the ready-to-use or dehydrated TBX media, provided by 4 different suppliers. These tests allowed the dehydrated PTX medium to be validated, but not the ready-to-use medium.

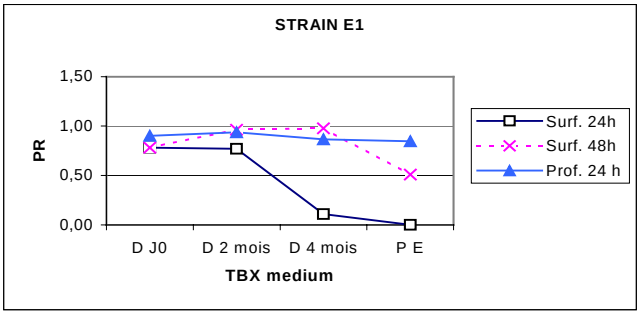
3.2.2 Productivity tests, realised in July 2004, on the strains used in CECALAIT's proficiency tests

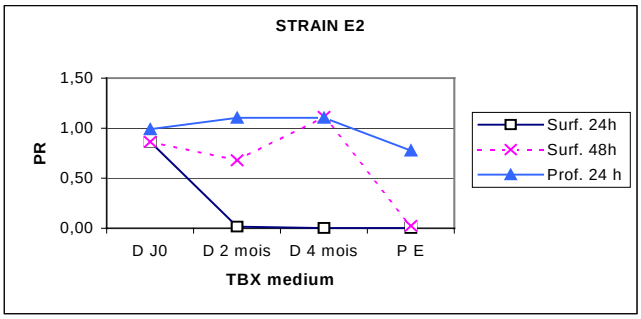
The objectives of this study, realised in the CECALAIT's laboratory, were to see:

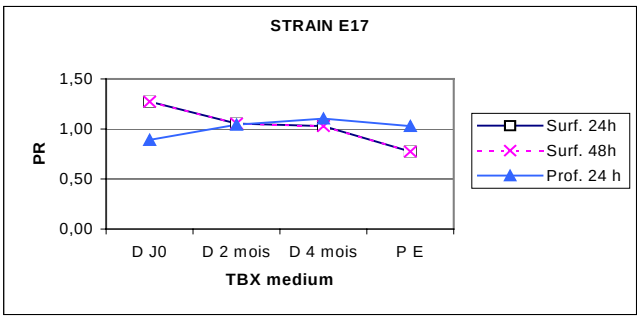
- if the strains used in the proficiency tests were sensitive to the medium effect,
- if productivity differences were obtained according to the media preparation mode, their time of storage and the inoculation, for the realisation of performance tests, on the surface or in the mass.

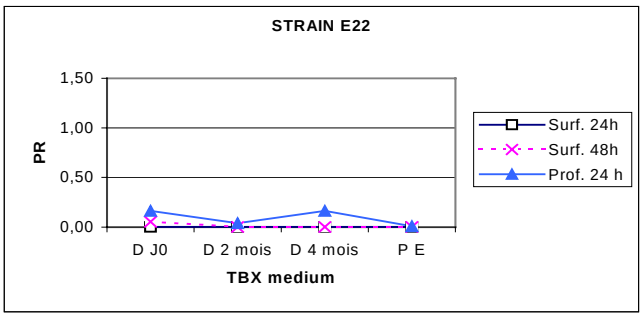
The 4 strains tested are usually used in CECALAIT's proficiency tests, and were isolated from samples of dairy products.
4 types of media, from a same supplier (A), were tested: a dehydrated TBX medium prepared the day of the study; the same medium used 2 and 4 months after preparation; ready-to-use TBX medium (PE); TSA-YE medium (Trypton-Soja-Agar and extract of yeast) used in the reference.
The dishes were inoculated on the surface (Spiral system) and in the mass, and incubated 24 and 48 hours at 44 ± 1°C. The productivity (PR) was calculated according to its different factors. The validation criteria of a batch was PR > 0.5.

The results presented in the figures below show the impact of different factors on the productivity of the medium TBX:









1- Strain effect: According to the strain tested, it can always be inhibited (E22), never inhibited (E17) or have a different response according to the media (E1 and E2). So, the strain E22 must be the same type as the strain, inhibited with all types of TBX, used in the study described below. The question is to know what is the frequency of this type of strain in naturally contaminated products. This could explain the problems met punctually in natural samples. Further to this study, the strain E22 is no longer used in CECALAIT's proficiency tests.

2- Medium effect: A medium effect is observed on strains E1 and E2. It appears that the media, classified in order of decreasing performance, are: - the dehydrated medium prepared the day of the study, - prepared 2 months before, - prepared 4 months before, - and the ready-to-use medium. Normally, the storage time of the prepared media must not exceed 3 months in the fridge (ISO/TS 11133-1). The performance of the medium TBX prepared in flasks decreases during storage and it must undergo additional heating before use. This could partially explain the deficiency of performance of the ready-to-use medium tested here.

3- Effect of inoculation type: The inhibition is more important on the surface than in the mass and allows obtention of more important medium effects. In the case of an inoculation on the surface, a longer incubation of 24 hours permits obtention of countable colonies, which, no doubt, did not develop or did not express the blue colour within 24 hours. This effect has already been observed in proficiency tests or in freeze-dried samples. Actually, the ISO/TS 11133-2 standard allows the choice concerning the type of inoculation. Chapter 5.3.1.2 note 2 indicates: "The pour plate method may also be used for culture media normally used for enumeration in this way". Therefore, the work group decided to propose a new, more direct wording for quantitative tests: the inoculation can be made on the surface or in the mass, but the protocol described in the standard corresponding to the tested medium must be used. This proposition was transmitted for approval to the work group in charge of the revision of the ISO/TS 11133-2 standard.

3.3 Inquiry on the realisation of performance tests in the laboratories:

- **Objectives of the inquiry:** The question was to know if these tests would be able to detect a batch which could cause problems during its use. Then, in September 2004, an inquiry was sent to the members of the Commission AFNOR V 08B. The purposes were to know if some problems were often met during the use of the medium TBX and if performance tests were realised often. In this case, do they permit to set aside batches for which problems were reported.

- **Results of the inquiry:** Only 6 laboratories on 95 answered. The problem most often met (3 laboratories) is the growth of other non-target bacteria. But, the use of ready-to-use media does

not seem to be the cause of this problem. Only one laboratory carries out performance tests, but it does not use the protocol and strains described in the standards. These tests could not underline the non-specificity problem observed by this laboratory in its routine analyses.

A priori, the standardised performance tests are not or rarely implemented in the laboratories using TBX. Only the suppliers realised them. At the end of these tests, which can not underline the problems described above, all the commercialised media comply.

4) CONCLUSION

Since the first usage of the medium TBX in substitution of the medium PTX for the enumeration of *E. coli* in food, several laboratories, analysing naturally contaminated samples or CECALAIT's proficiency test samples, punctually noticed inhibition problems on this medium. The bacteriostatic preservative in the proficiency test samples can produce an additional stress to the strains in relation to a natural sample, and so can accentuate the problems observed with certain batches.

The work group showed that effects due to various factors exist:
- the sample (type of strain, physiological condition of the strain, matrix),
- the media (batch, mode of preparation and storage),
- the method (diluent, mode of inoculation)…,
- and perhaps, other factors not yet identified.

No doubt, these effects added together can generate important differences in the enumeration.

Thanks to the work on the performance tests of media, the work group showed that these tests do not seem to be able to underline this type of inhibition and only a few user laboratories applied them.

However, this work group could not underline the origin of this problem, bound more to a variability of batches than to a particular supplier. The difference between the media PTX and TBX is essentially the result of their composition, which is different in peptones (meat peptone for PTX and casein peptone for TBX) and in selective agents (tergitol 7 for PTX and biliary salts n° 3 for TBX). The use of biological ingredients, such as peptones and biliary salts, can involve non negligible variations in culture. However, the ISO/TS 11133-2 (4.1.2) standard is tolerant in the quantity of ingredients implemented in the production of a batch of media. Therefore, the media suppliers optimise their formula. A bibliographical study (1) realised by a member of the work group shows that biliary salts can have, according to their nature, an inhibiting effect on *E. coli*. However, the biliary salts n°3, which compose the medium TBX are better defined chemically than classical biliary salts. They are also well purified and stable.

To solve this problem, the only conceivable issue was to work again on the composition and to propose a new media for standardisation. This proposition has been rejected for the moment by the work group, knowing that the problems on the medium TBX seem to be less frequently met. Moreover, this study will require a very important and long technical work supported by the member laboratories of the standardisation organisms.

As the work group "TBX" did not find the origin of this variability, its activity has been suspended, but we are staying tuned to users and, if need be, we will present another review in "La Lettre de CECALAIT" to inform you about new elements.

(1) If you want this bibliographical study, please contact us.

We would particularly like to thank:
- *the user laboratories which give us their results*
- *the members of the work group*