A Groundbreaking Culture Media Range To Simplify And Enhance Media Fill Tests Accuracy

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Good Manufacturing Practices (GMPs) require pharmaceutical companies to regularly perform Media Fill Tests (MFT) in order to verify the adequate microbiological state of their aseptic production processes for parenteral drugs. MFT are critical microbiological tests carried out to simulate the normal manufacturing conditions by replacing the pharmaceutical product by culture media. Manual reading of several thousands[3] of pharmaceutical containers filled with culture media is the current practice: it seems that for 62% of the pharmaceutical industry more than 5,000 units are filled each time, and for almost 25% more than 15,000 units are filled. Since each container must be individually checked, this step is extremely time-consuming and can be a source of potential errors: it requires highly qualified technicians, and time, to be performed in appropriate conditions. In this study we demonstrate that a new TSB culture medium approach for MFT with a color indicator optimizes and facilitates the reading step and can lead to less false reading errors. The new formulation was designed to meet the highest requirements from the pharmaceutical industry. Irradiated, cold filterable and animal free, this vegetable TSB was also optimized for the growth of anaerobic micro-organisms to reach the highest level of performances.

Production of parenteral drugs has always been considered as the most critical process in the pharmaceutical industry. To ensure sterility of these products, manufacturers use an aseptic filling process and must prove that this practice is under control. Media fills have become the standard approach to validate the protection from microbiological contamination provided by the aseptic filling procedure.

According to the European and US regulatory agencies, validation of an aseptic line must be realized with three successive successful Media Fill Tests (MFT), and each validated line has to be re-evaluated at least twice a year. MFT are critical for pharmaceutical companies because they have to be performed in the worst-case production conditions and each single unit has to be visually controlled after incubation. The culture medium that replaces the pharmaceutical product has to be carefully selected, it must show excellent growth performances and has to be safe and easy to use.

bioMérieux developed two groundbreaking ranges of culture media specifically designed for MFT. Both ranges comply with pharmacopoeias requirements for the growth of non-fastidious micro-organisms, including some anaerobic strains, are irradiated at least at 25kGy to ensure the absence of viable micro-organisms and mycoplasmas, and are cold filterable.

One of the range contains vegetable peptones instead of animal-derived peptones for "animal-free" facilities. Besides, considering the importance and the sensitivity of the MFT, bioMérieux innovates and includes in this vegetable formulation a growth-based proprietary color indicator to help for the reading of contaminated units. Contamination and turbidity can easily be revealed with an irreversible color change if growth occurs after incubation.

This study describes how both those new ranges of TSB 3P™ (Pharmaceutical Proven Performances) culture media with animal and vegetable peptones were designed and optimized to meet the highest requirements from the pharmaceutical industry.

Material And Methods

All the products tested in this study were dehydrated culture media. bioMérieux dehydrated Trypcase Soy Broth (TSB), reference 51019 was used as a positive growth control. The growth performance analysis was performed on the following references:

- bioMérieux Irradiated TSB 3P Animal Peptones, reference 51101 / 51102,
    - **TSB 3P AP**
- bioMérieux Irradiated TSB 3P Vegetable Peptones with color indicator, reference 51103 / 51104,
    - **TSB 3P VP**

Performances of different MFT media suppliers were compared to bioMérieux MFT media for the growth of anaerobic or micro-aerophilic micro-organisms:

- Becton Dickinson BBL Trypcase Soy Broth ref. 296264,
    - **Manufacturer A, batch 6335792**
- Oxoid Cold Filterable TSB ref. CM1065,
    - **Manufacturer B, batch 424880**

Culture Media Preparation

Culture media were prepared according to the respective suppliers instructions for use. Sterile filtrations were performed with a Stericup® 0.22µm, Millipore (reference SCGVU05RE). Media were aseptically aliquoted in 10ml.

Growth Performance Study

The growth promotion performances were checked on Pharmacopoeias strains and on clean room isolates. One hundred and one (101) micro-organisms were tested in this study (the list is presented in Table I):

- 80 aerobic bacteria,
- 6 anaerobic bacteria,
- 6 yeasts,
- 9 fungi.

Inocula were prepared from fresh micro-organisms culture by serial dilutions in a buffer sodium chloride solution pH 7; their final concentration was between 10 Colonies Forming Unit (CFU) and 100 CFU. The *inoculum* count was validated

on a Petri dish (TSA, bioMérieux reference 43011 or Columbia + 5% blood sheep, bioMérieux reference 43071).
Culture media were inoculated with 50 µl of each strain (4 tubes for each medium), then 2 tubes were incubated at 20-25°C and 2 tubes at 30-35°C during 14 days in aerobic or anaerobic conditions (for anaerobic bacteria).
Reading of the tubes was done every day and growth was measured according to an internal turbidity scale.

Color Indicator Performance Study
The evaluation of the TSB 3P VP media color change (vegetable peptones with color indicator) was only achieved on aerobic strains. Since the color indicator is a Redox chemical component, an anaerobic atmosphere induces its reduction and the spontaneous color change. Tubes were inoculated and incubated and turbidity was read according to the same protocol as described for the Growth Promotion Study. At the same time points, the color change of the indicator was also evaluated and the discoloration was rated under three (3) conditions: total, partial or no discoloration.

Filterability Study – V_{max} Determination
V_{max} (or V_{cap}) is the theoretical maximum volumetric throughput for a filter under a constant pressure. V_{max} was determined on 3 different filtering membranes:
- Polyvinylidene (PVDF), 0.22µm,
- Polyethersulfone (PES), 0.20µm,
- Nylon (NR), 0.22µm.

The test was repeated 10 times on each membrane.

Irradiation Cycle Validation
New bioMérieux MFT culture media were irradiated with a validated cycle between 25 and 40kGy. The irradiation study aimed at demonstrating the efficacy of the sterilization cycle on micro-organisms, and the absence of impact on media growth performances.
The killing validation was made using *Bacillus pumilus* ATCC 27142 biological indicators calibrated at $1.5x10^8$ CFU on strips. Strips were introduced in 500g and 5kg buckets of dehydrated culture media and were irradiated with a worst case condition of 25kGy dose (the lowest dose). Irradiated strips were then incubated in TSB culture media bioMérieux reference 44011 at 35°C ± 2°C during 7 days and the growth was controlled with the reading of turbidity.
Besides, growth performances of the media were controlled after irradiation as well. 500g and 5kg buckets of dehydrated culture media were irradiated with a worst case condition of 40kGy dose (the highest dose) and growth performances were then evaluated on a specific sample group of micro-organisms (see Table I).

Mycoplasma Absence
The "over-kill" effect of irradiation for mycoplasmas was validated by checking the absence of mycoplasmas before and after irradiation at 25kGy (lowest irradiation dose). This was controlled by an external third party laboratory who analyzed samples to check for the absence of the mycoplasmas described in the pharmacopoeia before and after the irradiation cycle:
- *Acholeplasma laidlawii* ATCC 23206
- *Mycoplasma gallisepticum* ATCC 19610
- *Mycoplasma hyorhinis* ATCC 17981
- *Mycoplasma orale* ATCC 23714
- *Mycoplasma pneumoniae* ATCC 15531
- *Mycoplasma synoviae* ATCC 25204

Two methods were used to detect mycoplasmas in the dehydrated media, with the protocol described in the following table:
- the indirect culture growth method with indicator cells
- a DNA and PCR-based method with a genetic amplification.

Time	Test
Day 0	Vero Cells growth
Day 2	Co-incubation of sample, positive control and negative control with Vero cells
Day 3	Vero cells subculture
Day 6	Vero cells subculture on slides; qPCR test
Day 7	Hoecht DNA staining and epifluorescence reading

Three (3) grams of dehydrated culture medium were mixed with 100 ml of de-ionized water, the solution was filtered on 0.45 µm and centrifuged in order to reduce the volume to extract. The sample (1 ml) was co-incubated with indicatives Vero cells at 37°C ± 2°C and in a 5% ± 1% CO_2 atmosphere.
Mycoplasma hyorhinis was introduced in a Vero cells flask as positive control and a NaCl 0.9% solution aliquot was also incubated with Vero cells as negative control.
Vero cells were removed from their 25cm² flask with trypsin at day 6, then DNA was extracted. PCR tests were performed with Q|Aamp DNA minikit (Qiagen). The detection was realized with generic primers which were able to reveal: *A. laidlawii, M. hyorhinis, M. orale, M. pneumoniae, M. synoviae.* An other specific primer was also used to detect *M. gallisepticum.*

Results and Discussion

bioMérieux Products Presentation
bioMérieux new dehydrated culture media TSB 3P AP and TSB 3P VP dedicated to Media Fill Test are available in two presentations: 500g powder in a plastic flask and 5kg double wrapped powder within a plastic bucket. Both packaging are tamper proof with an individual seal and the dehydrated media in their final packaging are irradiated, cold filterable and available with animal peptones or vegetable peptones. In the vegetable formulation, a red proprietary color indicator specific of microbial growth was added that gives to the reconstituted medium a red/pink coloration.

Raw Material Selection
A culture media contains delicate biological components in very precise quantity in the purpose to optimize the growth of micro-organisms. Several conditions directed the raw material selection:
- absence of mycoplasma,
- limited bioburden,
- good filterability of the peptones,
- high growth performances.

Each raw material was tested to be conform to specific criteria and validated to be used in the final media formulation. The bioMérieux TSB 3P AP and TSB AP VP were manufactured with TSE free peptones and all the raw materials entering in the composition of the final MFT media were controlled in pilot batches.

Growth Performances

MFT dehydrated culture media are exposed during the irradiation process to conditions (temperature, radiations…) that could damage their properties. The culture media growth promotion aims at verifying if both irradiated TSB 3P with animal or vegetable peptones specifically designed for MFT are equivalent to a non-irradiated TSB culture media. In order to verify the growth performance, the growth promotion test was performed on the bioMérieux TSB Media Fill culture media and with the TSB reference, with 101 strains, either ATCC strains or isolates from the production environment (see Figure 1 and Figure 2).

- ***Optimized Performances For Quick Aerobes Detection***

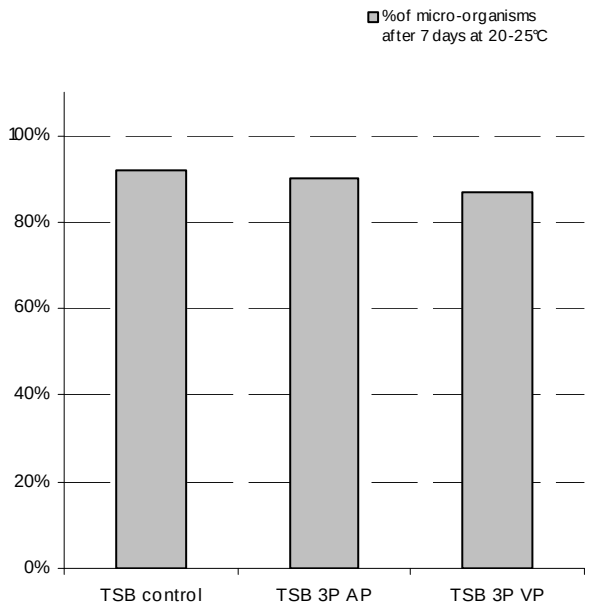

Fig 1: Proportion of all the strains that grew after 7 days of incubation at 20-25°C.

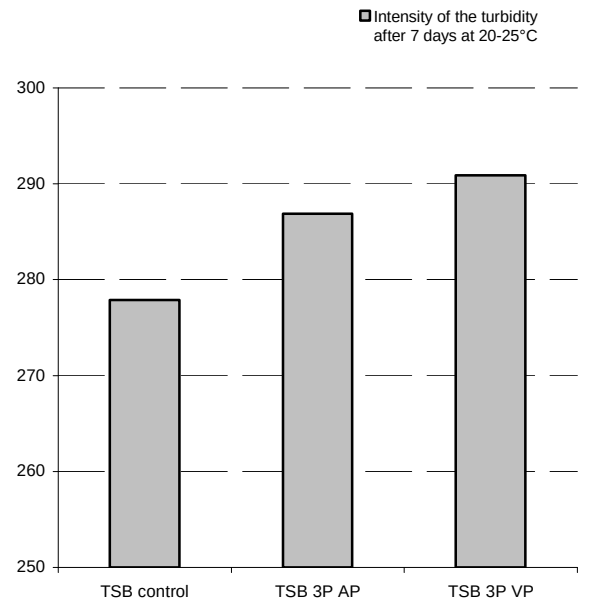

Fig 2: Growth promotion results on all strains after 7 days at 20-25°C. Turbidity values were summed for each micro-organism for each culture medium.

The results in Figure 1 clearly show that the TSB 3P AP and TSB 3P VP optimized for Media Fill Tests are equivalent to the non irradiated TSB control in terms of broad range detection.

Besides, even though the broad range detection is equivalent to the non irradiated TSB control, Figure 2 demonstrates the quicker micro-organism detection ability of TSB 3P AP and TSB 3P VP. Both those media for Media Fill Test were optimized to get a stronger turbidity, therefore to get an easier reading of the contamination.

As shown in those 2 figures, all data obtained during the growth promotion test support the fact that the irradiation process doesn't have any effect on the performances of the TSB 3P AP and TSB 3P VP for MFT, and that the quick detection performances were optimized compared to the reference.

- ***Optimized Performances For Quick Anaerobes Detection***

Even though research of anaerobic micro-organisms is only required in anaerobic process conditions, a lot of aseptic filling are performed under nitrogen generating an atmosphere poorer in oxygen.

This is why both TSB 3P AP and TSB 3P VP were specially designed and optimized to grow anaerobic bacteria as shown in the Figures 3.

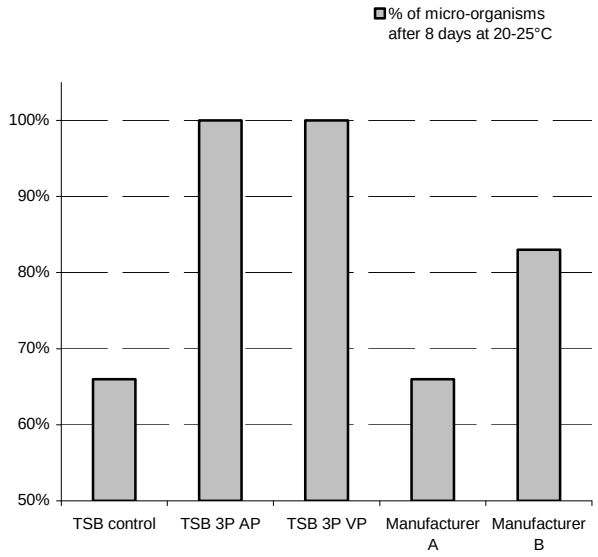

Fig 3: Proportion of all the anaerobic strains that grew after 8 days of incubation at 20-25°C.

The results in Figure 3 clearly show that in the conditions of the experiment, the TSB 3P AP and TSB 3P VP media optimized for MFT have higher performances than the non irradiated TSB control and the other manufacturers. 100% of the anaerobic strains grew in TSB 3P AP and TSB 3P VP after 8 days of incubation at 20-25°C.

Besides, the intensity of the turbidity was also controlled in this experiment to check that the growth was clearly visible (see Figure 4).

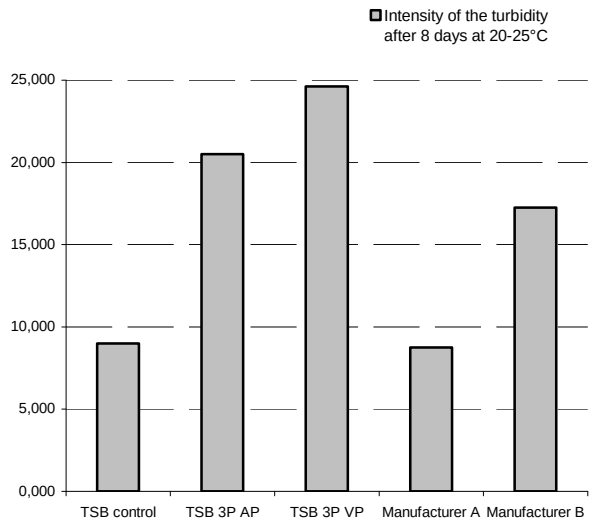

Fig 4: Growth promotion results on all anaerobic strains after 8 days of incubation at 20-25°C. Turbidity values were summed for each micro-organism for each culture medium.

In the conditions described, those results confirm the optimization for specific anaerobic micro-organism detection and the superiority of bioMérieux new TSB 3P range for Media Fill compared to the other products.

Besides, in this specific experiment, no growth was observed for the other manufacturers for *Propionebacterium acnes* ATCC 6919 at 4 days at 20-25°C. This isolate is a very common isolate from human skin, and personnel contaminants is for 70% of the pharmaceutical industries the most likely potential source of contamination in process simulations[3]. Only TSA 3P AP and TSB 3P VP were able to grow this isolate in the described conditions at 4 days, showing the higher performance of both those media compared to other suppliers.

Those results clearly illustrate that TSB 3P AP and TSB 3P VP were optimized to be adapted for efficient and quick anaerobe growth: an early detection of anaerobic micro-organisms in anaerobic conditions occurs during the first part of the incubation at 20-25°C. According to literature, such type of anaerobic testing is done in more than 15% of the cases[3] (with a Nitrogen gasing phase for example).

Color Indicator Study
bioMérieux MFT culture media references 51103 and 51104 (with vegetable peptones) contain a Redox color indicator. The reconstituted culture medium has a red color that is discolored with microbial growth. This indicator was selected because of its ability to be reduced by a very broad range of micro-organisms metabolism and to display an irreversible discoloration.
The discoloration study was done on the 95 aerobic strains. Out of the 95 tested strains, 92 had discolored the media. Only 3 strains (3,2%) never metabolized the indicator after 14 days at 20-25°C or 30-35°C:
- *Methylobacterium mesophilicum* bMx 0306751,
- *Corynebcaterium jeikeium* bMx 8710127,
- *Kocuria rosea* bMx 005008,

however their growth was clearly visible and the other strains of *Corynebacterium* and *Kocuria* tested still metabolized the color indicator with the irreversible color change.

The results of this study illustrate that the chosen color indicator is well adapted for a growth indicator use. After a 14 days of incubation, 100% of the strains grew and 97% discolored the culture medium

Irradiation Cycle Validation
In order to guarantee the total absence of viable micro-organisms in the dehydrated media, irradiation validations were performed to validated the process, and the reproducibility of the results. The absence of growth of the *B. pumilus* $1{,}5x10^8$ CFU biological indicators after a 25kGy irradiation establishes a 8 log killing condition. In accordance with the raw material bioburden analysis (data not shown), the irradiation process ensures a dehydrated culture media for Media Fill Tests free of any viable micro-organisms.
Complementary growth promotion tests were also realized after a 40kGy irradiation. The media performances were checked with a restricted micro-organisms sample group and the results (data not shown) demonstrated that no performance differences were observed between the irradiated and the non irradiated media.

Mycoplasma Absence
Mycoplasmas is the common term to designate the Mollicutes group, they are parasites of animals and plants cells; however, the genus *Mycoplasma* is by definition restricted to vertebrate hosts. Mycoplasmas are characterize by a lack of cell wall and are physically small – less than 1µm –; that give them the ability to pass through filters. Their presence was tested inside the dehydrated culture media before irradiation in order to verify the level of the initial contamination. Three different lots with different peptones batch numbers of both medium (animal and vegetable peptones) were tested. No mycoplasma were found in the powder (data not shown) that plainly establishes the high quality of the raw material used in the MFT media manufacturing.
The absence of mycoplasma was also confirmed after a 25kGy irradiation cycle (lowest dose). Mycoplasma were never detected in any of the six different culture media batches tested.

Filterability Study
The animal peptone formulation showed a similar range of filterability with the different types of membranes. The animal formulation is more filterable due to the fact that animal peptones are more fluid.
However, it was demonstrated by a real case study in the pharmaceutical industry that a 4,000 liter TSB 3P VP media preparation was finally filtered on 0,22µm without any clogging problem. The conditions were with a pre-filtration on 0,2µm and a 2.5 Bar pressure.

Conclusion

Media Fill Tests are a necessary part of aseptic processes validation and on-going periodic monitoring. The number of units filled in a MFT should be determined by the risk analysis of the aseptic production process and can reach more than 15,000 units for almost 25% of the pharmaceutical industry[3] and the common lower limit is 5,000 unit per trial[1]. The manual reading of every single container is a real bottleneck because of the reading time and also for the risk of reading errors.

In this study, bioMérieux presented a breakthrough improvement for the MFT reading accuracy with a new irradiated cold filterable TSB medium, containing vegetable peptones and a unique color indicator. This product was developed with selected raw materials to guarantee the culture medium to be TSE free and to be free from any viable micro-organism and mycoplasma.

The TSB 3P VP performances meet the highest requirements with a positive growth of all the 101 culture collection strains and environmental isolates tested in the study, and with an irreversible color change of the indicator in 97% of the cases. The presence of the color indicator facilitates the visualization of the micro-organisms growth.

The study also illustrates the optimization and the ability of the TSB 3P culture media to recover the anaerobic strains from environment which can occur in about 3% of the times[3].

According to the probabilities of events that could contaminate an aseptic process[2,3], each MFT would theoretically need to be run on as many as 100,000 to 400,000 units to be able to detect such a very low contamination rate. That was clearly unrealistic with the available solutions in the past. Now, with the color indicator for easier and safer reading of MFT units, batch sizes could be increased to be more representative. And in the future, with an automatic reading of the units, the growth color indicator breakthrough might allow to increase the size of the MFT batch to be even more similar to a normal production batch and then be more confident about the sterility assurance limits.

References

(1) Guidance for Industry - **Sterile Drug Products Produced by Aseptic Processing** - Pharmaceutical cGMPs, FDA 2004.

(2) Nigel A. Halls, **Practicalities of setting acceptance criteria for media fill trials**. PDA Journal of Pharmaceutical Science and Technology, 2000 ; **54**, 247-252.

(3) PDA Technical Report No. 36, **Current Practices in the Validation of Aseptic Processing – 2001**. PDA Journal of Pharmaceutical Science and Technology, May-June 2002; supplement TR36, Vol. 56, Number3.

Table I: Micro-organisms tested in the study.
Highlighted strains are described in Pharmacopoeias
Strains in bold represent the restricted sample group.

Acinetobacter lwoffi	bMx 7604328
Acinetobacter baumanii	bMx 21925
Actinomyces neuii spp anitratus	bMx 0605704
Acremonium	bMx 0602720
Aerococcus viridans	bMx 0011701
Aeromonas hydrophila	ATCC 7965
Alcaligenes faecalis	ATCC 8750
Alternaria alternata	bMx 0710714
Aureobacterium spp Corynebacterium aquaticum	bMx 0502715
Aspergillus niger	**ATCC 16404**
Aspergillus fumigatus	bMx 1031
Bacillus cereus	ATCC 7064
Bacillus circulans	bMx 0506750
Bacillus coagulans	bMx 0105700
Bacillus licheniformis	bMx 0506752
Bacillus megaterium 1	bMx 0506753
Bacillus pumilus	bMx 0011703
Bacillus sphaericus	bMx 0405750
Bacillus subtilis	**ATCC 6633**
Bacillus mucoïdes	bMx 0611704
Bacteroides fragilis	ATCC 25285
Bacteroides vulgatus	**ATCC 8482**
Brevibacillus brevis	bMx 0010703
Brevibacterium epidermidis	ATCC 14869
Brevundimonas vesicularis	bMx 0306754
Burkholderia cepacia	ATCC 25416
Candida albicans	**ATCC 10231**
Candida krusei	bMx 1303
Candida famata	bMx 8706052
Candida glabrata	bMx 9911601
Cellulomonas spp/Microbacterium spp	bMx 0609700
Citrobacter freundii	ATCC 8090
Citrobacter koseri / amalonaticus	bMx 0710712
Chryseobacterium indologenes	bMx 0605705
Chryseomonas indologenes	bMx 0306756
Clostridium sporogenes	**ATCC 19404**
Clostridium perfringens	ATCC 12916
Comamonas acidovorans	bMx 0306750
Corynebacterium jeikeium	bMx 8710127
Corynebacterium striatum	bMx 0503059
Corynebacterium xerosis	bMx 0509712
Cryptococcus neoformans	ATCC 32045
Enterobacter aerogenes	bMx 8710080
Enterobacter cloacae	ATCC 13047
Enterococcus durans	bMx 545
Enterococcus faecalis	**ATCC 29212**
Enterococcus gallinarum	bMx 9812094
Escherichia coli	**ATCC 8739**
Escherichia coli	ATCC 25922
Escherichia fergusonii	bMx 0602754
Flavimonas oryzihabitans	bMx 0012700
Fusarium	bMx 0611727
Geotrichum candidum	bMx 6401
Hafnia alvei	bMx 12905
Klebsiella pneumoniae	ATCC 13883
Kocuria kristinae	bMx 0406750
Kocuria rosea	bMx 0005008
Lactobacillus casei	IM 477
Leclercia adecarboxylata	bMx 0305702
Listeria monocytogenes	ATCC 19111
Methylobacterium mesophilicum	bMx 0306751
Microbacterium spp/Leifsonia aquatica	bMx 0710704
Micrococcus luteus	**ATCC 9341**
Micrococcus spp	bMx 0306755
Micrococcus lysodeikticus	bMx 1456
Ochrobactrum anthropi	bMx 0610700
Pediococcus damnosus	ATCC 29358
Penicillium italicum	ATCC 48114
Phoma	bMx 0611717
Propionibacterium acnes	**bMx 9910602**
Propionibacterium acnes	ATCC 6919
Proteus mirabilis	ATCC 12453
Providencia stuartii	bMx 1207
Pseudomonas aeruginosa	**ATCC 9027**
Pseudomonas fluorescens	bMx 8509114
Pseudomonas putida	bMx 0310750
Pseudomonas stutzeri	bMx 0610701
Ralstonia picketti	bMx 0306753
Rahnella aquatilis	ATCC 33071
Raoultela planticola	bMx 9701066
Rhizobium radiobacter	bMx 0703700
Saccharomyces cerevisiae	ATCC 9763
Salmonella abony	bMx 80-39
Salmonella arizonae	ATCC 12325
Scopulariosis	bMx 0611728
Serratia marcescens	ATCC 13185
Shewanella putrefaciens	bMx 0509717
Shigella flexneri	ATCC 12022
Sphingomonas paucimobilis	bMx 0404755
Staphylococcus aureus	**ATCC 6538**
Staphylococcus epidermidis	**ATCC 14990**
Staphylococcus hominis	ATCC 27845
Staphylococcus saprophyticus	bMx 0405751
Staphylococcus warnerii	bMx 0405752
Staphylococcus xylosus	bMx 20266
Stenotrophomonas maltophilia	bMx 0409759
Stomatococcus mucilaginosus	bMx 0606700
Streptococcus pyogenes	**ATCC 19615**
Streptococcus uberis	bMx 0710700
Trichophyton mentagrophytes	ATCC 9533
Yersinia enterolitica	ATCC 23715

(bMx: bioMérieux environmental isolates)