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*Listeria monocytogenes*
http://eurl-listeria.anses.fr

# A new surface inoculation technique using an airbrush to perform challenge tests for *Listeria monocytogenes*:

# Results of preliminary tests

*Version 1 – 29/01/2015*

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# 1 INTRODUCTION

The EURL Lm has convened a NRLs working group (WG) in charge of the revision of the EURL *Lm* Technical Guidance Document for conducting shelf-life studies. This WG had identified the need for investigating and recommending new techniques for the initial contamination of food samples. One of the most important aspects was to improve the simulation of the real (natural) contamination.

In the version 3 (06/06/2014) of the EURL *Lm* Technical Guidance Document, some examples of initial contamination techniques are provided and the standard deviation related to the initial contamination level is expected to be lower or equal to 0.5 $\log_{10}$ cfu/g. But, despite the fact that contamination with an airbrush is becoming more largely used, this contamination technique is not cited in this version, by lack of data concerning the precision of this method at the moment of diffusion.

In this frame, EURL *Lm* has conducted a study of initial contamination with an airbrush in comparison with a contamination by spots on agar surface, the main goal being to collect data to assess the performance of the airbrush technique.

# 2 PRESENTATION OF AIRBRUSH

## 2.1 DESCRIPTION

The airbrush kit, model Aztek A430, comprises 3 nozzles each with a different size (a gray nozzle/40mm, a turquoise nozzle/50mm and a red nozzle/53mm), a nozzle wrench, a gravity feed cup, 2 siphon caps, a compressor adapter and an air compressor.

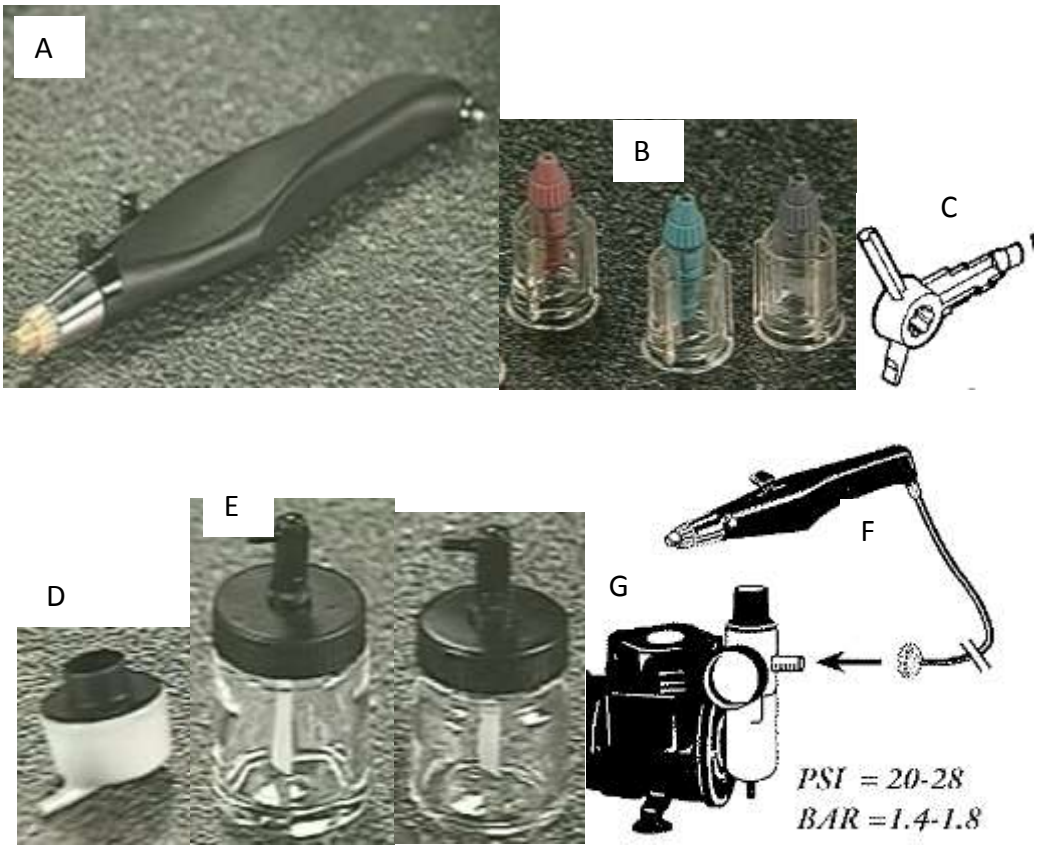


**Figure 1. Description of the Aztek A430 airbrush kit: (A) airbrush, (B) nozzles, (C) nozzle wrench, (D) gravity feed cup, (E) siphon caps, (F) compressor adapter and (G) air compressor.**

### 2.1.1 DETERMINATION OF FLOW

For spraying liquid from airbrush, it is necessary to use the trigger. In order to have a fixed flow, it is necessary to have a fixed stop. The initial stop of the airbrush allowed spraying a too big quantity of liquid in a too fast time. So a new stop was created to obtain a flow corresponding to our expectations.

This new stop deleted the possible use of the red nozzle.

The tightening of the nozzle having an impact on the flow, it is necessary to tighten it at the maximum and then to loosen the equivalent of a notch, in order to standardise the flow.

The flow of the 2 other nozzles was measured.

Table 1. Flows of the gray and turquoise nozzles

| Nozzles | Flow (ml/s) | Flow (seconds for 1 ml) |
|---|---|---|
| Gray nozzle | 0.017±0.002 | 59.3±6.6 |
| Turquoise nozzle | 0.043±0.004 | 23.7±2.3 |

### 2.1.2 PRELIMINARY CONTROLS

A non-pathogenic strain, a strain of *Lactobacillus sakei*, was used to perform these experiments.

#### 2.1.2.1 CONTROL OF AIRBORNE CONTAMINATION

Bacterial experiments were performed under a microbiological safety cabinet. An air sampler for microbiological monitoring was used to check the absence of pulverisation of bacteria by the airbrush in the atmosphere of the safety cabinet (monitoring of 100 litres) and so only towards the targeted area. No bacterion grew on non-selective agar. This experiment was repeated three times.

#### 2.1.2.2 CONTROL OF AIRBRUSH DISINFECTION

Table 2. Description of the controls to avoid cross-contamination, implemented on the airbrush kit

| Control period | Methods | Results |
|---|---|---|
| Before each use | Dipping of the head of the airbrush (the slide on contact with the nozzle), gravity feed cup and nozzle during few seconds into successive bathes: alcohol 70° then distilled water and finally diluent | |
| Before the 1st pulverisation | Pulverisation of 200 µl of diluent through the assembled airbrush on a non-selective agar | No colony |
| After each use | Dipping of the head of the airbrush (the slide on contact with the nozzle), gravity feed cup and nozzle during different times into successive bathes: disinfectant (Surfanios or Amphospray) during 3 different tested times (15, 20 and 30 minutes), then during few seconds for alcohol 70° and distilled water. | 1 colony after 15 min, 2 colonies after 20 min, no colony after 30 min in disinfectant bath (see Figure 2). Conclusion: an at least 30 minutes dipping period for disinfectant bath |

This experiment was repeated twice.

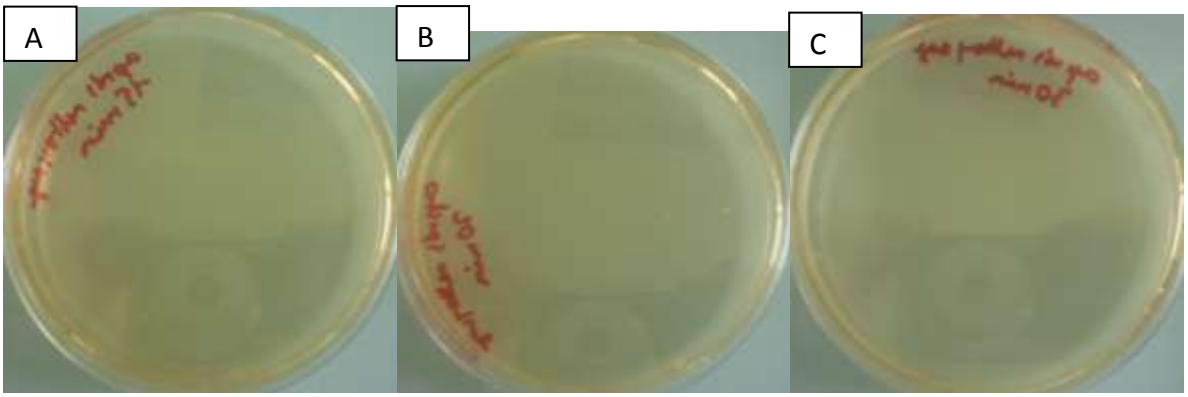


**Figure 2. Results of the disinfectant bath according to the duration (A: 15 minutes; B: 20 minutes and C: 30 minutes)**

### 2.1.3 PRELIMINARY TESTS

Before using bacteria to test the airbrush, a dye, methylene blue, was used to study in particular the nozzles, the appropriate pulverisation distance, the choice of the more appropriate cap.

**Table 3. Preliminary tests to improve the use of the airbrush kit**

| Tested parameters | Observation | Conclusion |
|---|---|---|
| Pulverisation speed according to the used nozzle | A too fast liquid pulverisation with the turquoise nozzle | The gray nozzle is retained |
| Pulverisation distance | An unappropriated pulverisation distance at 10 cm because of an observed lack of pulverisation precision | A 5 cm pulverisation distance is retained |
| Choice of the more appropriate cap | Not the choice of the siphon caps because of the pulverisation of the partial quantity of liquid | Choice of the gravity feed cup because of the pulverisation of the whole quantity of liquid |

### 2.1.4 TESTS WITH BACTERIA ON NON-SELECTIVE AGAR

#### 2.1.4.1 COMPARISON WITH CLASSICAL SPREADING

Systematically, the pulverisation of the bacterial suspension was compared to a classical spreading, with a spreader at the agar surface.

On 90-mm Petri dishes containing TSAYe, we distributed for each method 300 µl of the appropriately diluted bacterial suspension.

Each experiment was repeated twice and reproduced twice.

**Table 4. Comparison between a classical spreading and a pulverisation spreading**

| Methods | Results of the 1st reproduction | Results of the 2nd reproduction |
|---|---|---|
| Classical spreading | 52 and 56 cfu | 84 and 90 cfu |
| Pulverisation spreading | 56 and 100 cfu | 72 and 87 cfu |
| Conclusion | Equivalent enumeration and dispersion on TSAYE surface (see Figure 3) | |

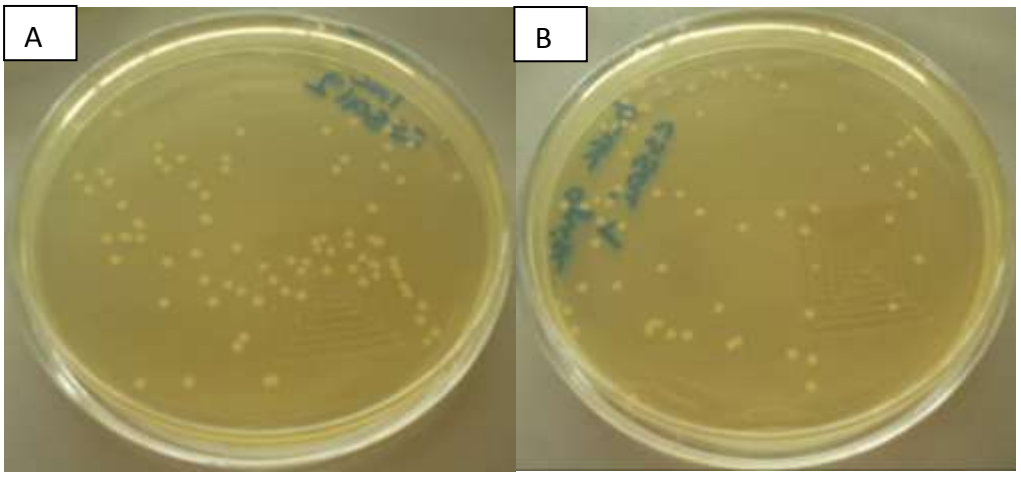


**Figure 3. Repartition of colonies on the TSAYe surface (A: classical spreading and B: pulverisation spreading)**

Moreover, four experiments were performed with 140-mm Petri dishes containing TSAYe and a targeted area corresponding to a 90-mm diameter circle. The aim was to know if the pulverisation is well-oriented.

**Table 5. Observation of the dispersion by using the pulverisation spreading**

| Method | Number of colonies in the targeted area | Number of colonies outside the targeted area |
|---|---|---|
| Pulverisation spreading | 80, 59, 53 and 73 cfu | 4, 7, 6 and 3 cfu |
| Conclusion | The percentage of colonies outside the targeted area is between 4% and 11%. (see Figure 4) | |

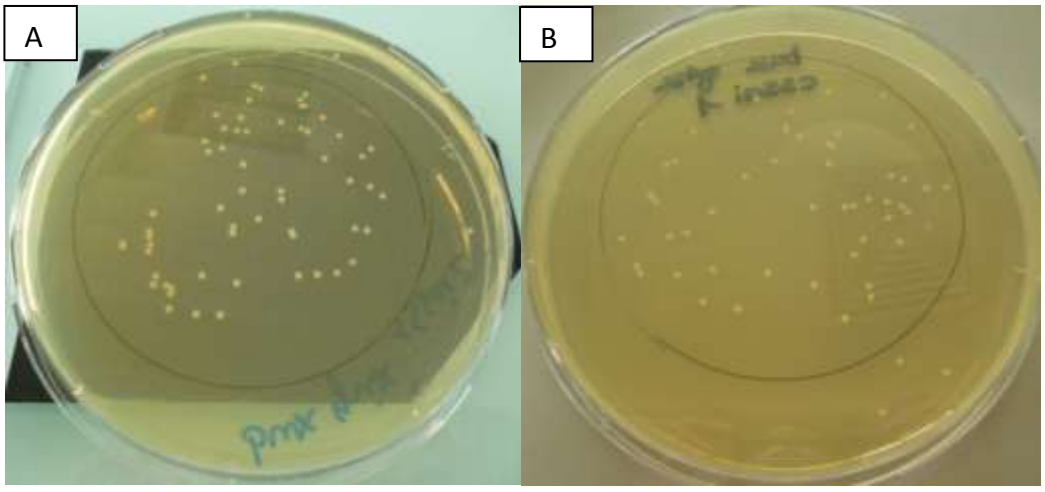


**Figure 4. Precision of pulverisation in a targeted area (A: 4 colonies out the targeted area and B: 3 colonies out the targeted area)**

### 2.1.4.2 ADEQUATE PULVERISATION VOLUME BY THE AIRBRUSH

It is interesting to be able to pulverise small volumes of bacterial suspension, while keeping the targeted value of initial concentration, without modification of the water activity of the tested matrix. As written in the 3rd version of the EURL *Lm* technical guidance document, it is necessary to respect a maximum of 1% between the inoculum volume and the mass or volume of the matrix.

For the trials on 90-mm Petri dishes, a reference bacteria concentration with a 100 µl classical spreading (with a spreader at the agar surface) was $8.7.10^8$ cfu/ml. The pulverisation technique was tested to compare with the former technique, by spreading 100 µl, 200 µl and 300 µl at different agar surfaces on 90-mm Petri dishes. The results and the conclusion are detailed in Table 6.

**Table 6. Adequate pulverisation volume according to the surface to be contaminated on 90-mm Petri dishes**

| | Pulverisation volumes spread on 90-mm Petri dishes | | |
|---|---|---|---|
| | 300 µl | 200 µl | 100 µl |
| Concentration obtained by pulverisation spreading | $1.10^9$ cfu/ml | $1.2.10^9$ and $9.8.10^8$ cfu/ml | 5.6 and $7.2.10^8$ cfu/ml |
| Conclusion | Satisfactory precision. Too fast pulverisation with 100 µl in order to allow a good repartition on the contaminated surface. The volume of 200 µl was retained for 2 main reasons: a not too fast pulverisation and a not too important adsorption volume. | | |

For the trials on large agar plates (38x28 cm), the pulverisation technique was used to spread 1 ml, 2 ml and 6 ml. Reference bacteria concentrations obtained by classical spreading (100 µl of the suspension at the agar surface on 90-mm Petri dish) were respectively $6.2.10^8$ cfu/ml and $6.8.10^8$ cfu/ml. These enumerations were compared to the ones obtained by spreading 1 ml and 2 ml by pulverisation technique. The results and the conclusion are detailed in the Table 7.

**Table 7. Adequate pulverisation volume according to the surface to be contaminated on large agars**

| | Pulverisation volumes spread on large agars (38x28cm) | | |
|---|---|---|---|
| | 1 ml | 2 ml | 6 ml (maximum volume of the studied gravity feed cup) |
| Concentration obtained by pulverisation spreading | $4.8.10^8$ cfu/ml | $5.0.10^8$ cfu/ml | |
| Conclusion | Correct concentration in comparison to the one obtained by classical spreading | Correct concentration in comparison to the one obtained by classical spreading. No difference between results obtained with the volumes 1 ml and 2 ml, so equal use of these 2 volumes (see Figure 5) | Volume not appropriate to respect the EURL Lm technical guidance document |

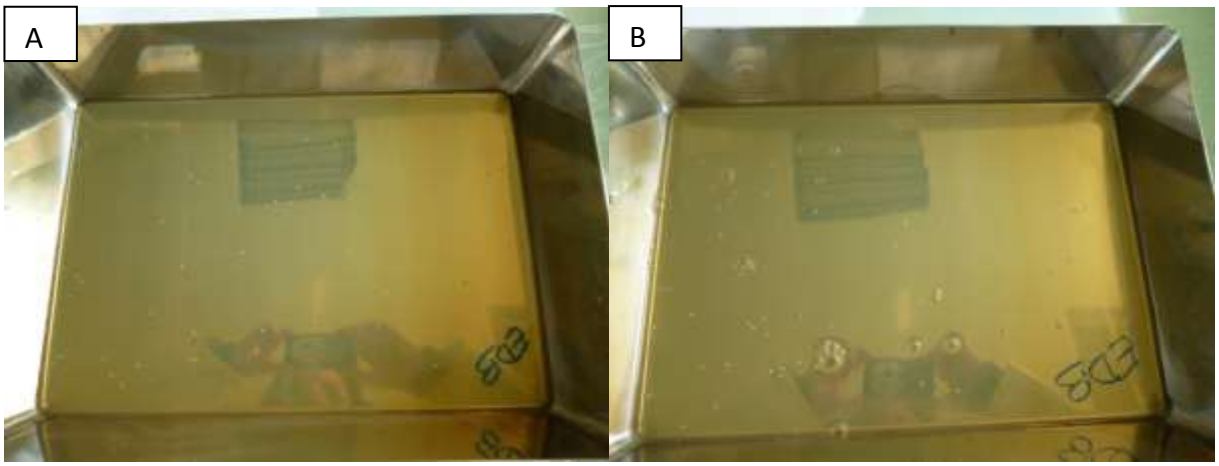


**Figure 5. Comparison between 2 pulverisation volumes (A: 1 ml and B: 2 ml) on large agars (28x38cm).**

### 2.1.4.3 POSSIBILITY TO PULVERISE A PRECISE SMALL QUANTITY OF BACTERIA

One of the aims of the study was to determine whether it was possible to pulverise a very precise small quantity of bacteria.

The pulverised volume was fixed at 200 µl. The aim was to reach 5 or 15 cfu on the surface of a 90-mm Petri dish.

**Table 8. Possibility to pulverise a precise small quantity of bacteria**

| Targets | 5 cfu | 15 cfu |
|---|---|---|
| Results | 7, 1, 4, 6, 2 and 3 cfu<br>(see Figures 6.A and 6.B) | 11, 12, 10, 14, 9 and 15 cfu<br>(see Figures 6.C and 6.D) |
| Conclusion | Possible use of pulverisation technique to contaminate a matrix at a precise small bacterial concentration | |

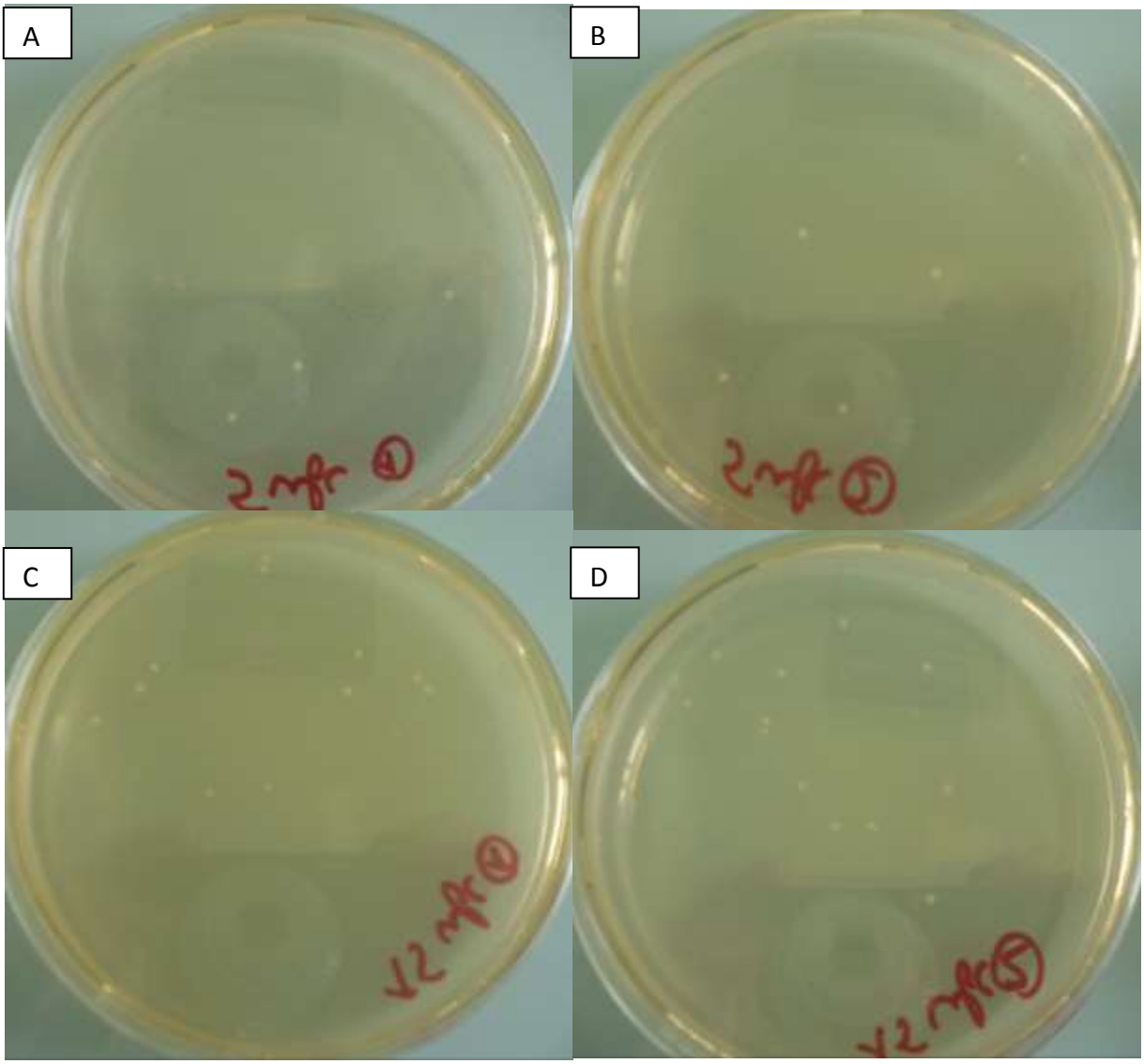


**Figure 6. Small numbers of colonies obtained thank to the airbrush (A: 4 cfu versus target of 5 cfu, B: 6 cfu versus target of 5 cfu, C: 11 cfu versus target of 15 cfu and D: 12 cfu versus target of 15 cfu)**

#### 2.1.4.4 COMPARISON BETWEEN TWO MATRIX CONTAMINATION TECHNIQUES

The technique currently used at the laboratory to contaminate a slice of smoked salmon is to take 100 µl of the inoculum at the appropriate dilution, then to deposit on half of the slice five spots of 20 µl, to fold over the other half of the slice and to use a spreader to improve spreading of the inoculum on the matrix.

This current technique on smoked salmon was compared to the pulverisation technique on TSAYe.

**Table 9. Comparison between two matrix contamination techniques**

| Methods | Deposit of 5 spots of 20 µl on a targeted area without spreading (see Figure 7.A) | Deposit of 5 spots of 20 µl on a targeted area with spreading (see Figure 8.A) |
|---|---|---|
| Classical spreading | $6.4.10^8$ cfu/ml | $5.5.10^8$ cfu/ml |
| Pulverisation of 100 µl on a targeted area (see Figures 7.B and 8.B) | $5.6.10^8$ cfu/ml | $5.3.10^8$ cfu/ml |
| Conclusion | Slightly larger number of colonies with the classical spreading<br>Hypothesis: less loss of the colonies on material surfaces with the classical spreading because of a unique contact with the tip of the pipette versus contact with the tip of the pipette, the gravity feed cup and the airbrush with the pulverisation spreading | An equivalent number of colonies with both techniques and a correct repartition of these colonies on the limited area.<br>Drawback of the classical technique with spreading: spreading not always possible, according to the studied matrix. |

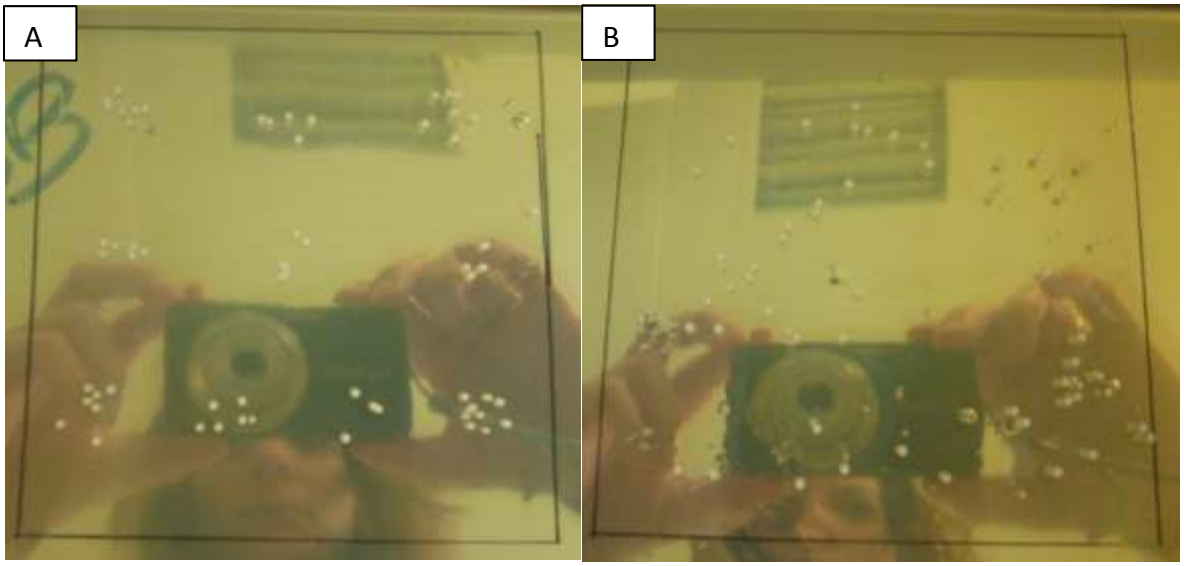


**Figure 7. Comparison of 2 contamination techniques (A: deposit of 5 spots without spreading and B: pulverisation of the suspension)**

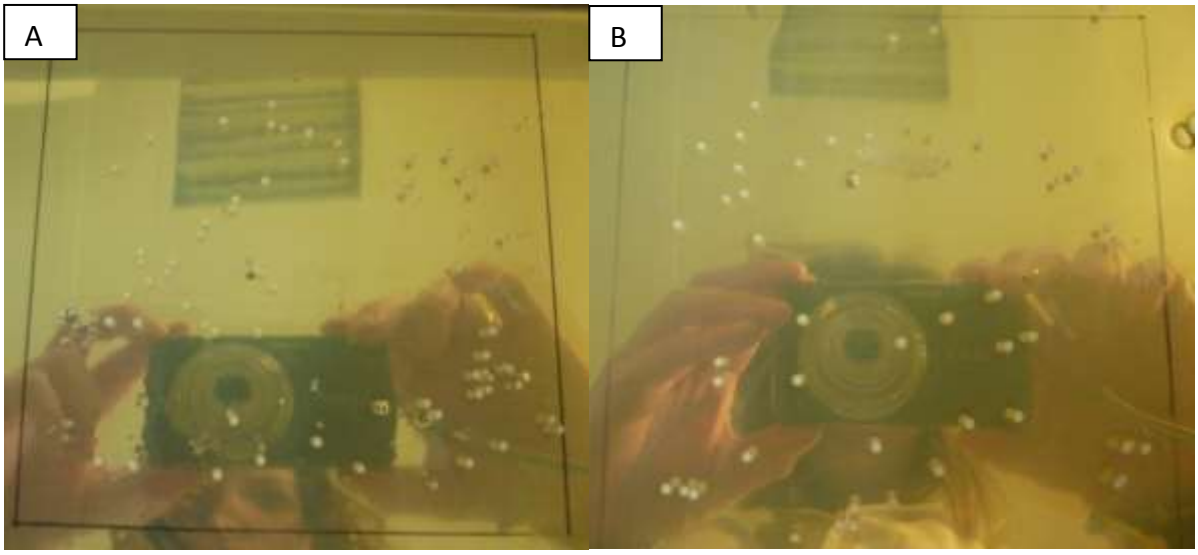


**Figure 8. Comparison of 2 contamination techniques (A: deposit of spots with spreading and B: pulverisation of the suspension)**

# 3 CONCLUSION

The contamination technique by airbrush pulverisation can be used for artificial contamination in the frame of challenge tests as well as PT trials, for *L. monocytogenes* as for other bacteria.

In 2015, this technique will be implemented by EURL Coagulase positive staphylococci to contaminate solid food matrices in the frame of the organisation of future PT trials so as to optimise the artificial surface contamination. Thus, this technique could help EURL and NRLs to implement easily an artificial contamination technique, while improving the repeatability and ensuring a satisfactory homogeneity and stability of CPS contamination.

In addition, according to the results obtained, a sub-sampling step of the test portion could be included and so performed by the participating laboratories, in particular during a PT trial on enumeration. Indeed, it is important to include this initial step of the analysis in PT trials, as it can have a major impact on the validity of the analyses of solid matrices.