SAMPLING TECHNIQUES FOR MICROBIOLOGICAL ENVIRONMENTAL MONITORING IN THE FOOD INDUSTRY

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1.- Microbiological environmental monitoring and food safety

Microbiological monitoring programs play a crucial role in the verification of the effectiveness of implemented hygiene control measures as defined in prerequisite and operational prerequisite programs as well as in HACCP plans.

The safety of foods can only be ensured through the implementation of effective preventive measures and cannot be achieved just by analyzing finished products. Testing of finished products for a pathogen is clearly not sufficient to guarantee their safety as low but significant levels of pathogens may not be detected even when sampling and testing large number of samples.

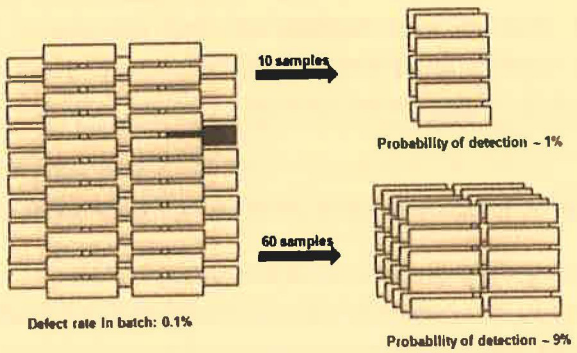

Figure 1: Statistical limitations of finished product testing (From ICMSF 2002, Volume 7)

Microbiological monitoring plays an essential role to detect deviations such as presence of pathogens in processing environments or increasing levels of hygiene indicators as early as possible. This allows to rapidly take corrective actions to ensure the safety and compliance of finished products.

Microbiological monitoring programs are used to verify the effectiveness of the implemented hygiene control measures. They are composed of the following four elements:

- Raw materials;
- Environmental samples;
- Line samples;
- Finished products.

Environmental sampling aims at detecting as early as possible the presence of pathogens or deviations of hygiene indicator beyond defined limits in order to rapidly apply corrective actions. These samples may be subdivided according to a priority rating which reflects the impact on processing lines and product in case of contamination:

- Samples taken from external surfaces of equipment where **a direct contamination** of the processing line or even of the product could easily occur.

- Samples taken from sampling sites from areas **further away from the processing lines** or exposed products, where presence of a pathogen could easily lead to its presence in locations close to the product or processing line.
- Samples taken from sampling sites from **very remote areas** and therefore no risk for processing lines and product. Results from such samples may provide information on specific niches or in case of investigations.

A correct selection of sampling sites and sampling tools is critical to assure the quality of the subsequent analysis and results.

2.- Enviromental samples

Complete absence of pathogens and levels of hygiene indicators consistently below established limits are certainly the target to ensure the food safety of products. Such results may however also reflect a poor microbiological monitoring program, inappropriate sampling procedures, inappropriate conditions during transportation of samples, their handling and testing. Such results need therefore to be challenged and procedures scrutinized carefully to determine whether there are weaknesses and identify opportunities for improvement.

To be representative and useful, sampling must be done taking the following elements in consideration:

- Correct timing reflecting the worst case situation such as build up;
- Correct timing to avoid a bias caused by cleaning and "hiding" the real situation;
- Correct timing to avoid affecting results by residues of cleaning agents or disinfectants;
- Details on locations where build can take place;
- Appropriate tools to recover contaminated residues.

3.- Surface sampling methods

The purpose of sampling of surfaces in food industry is to determine the presence of, or the number of, viable microbes on the surfaces of utensils, work surfaces and other equipment in contact with food to estimate the level of contamination during production, or the effectiveness of cleaning and disinfecting protocols.

Most typical surface sampling methods are the contact plates and the swab method, both included in the ISO 18593:2004. "Horizontal methods for sampling techniques from surfaces using contact plates and swabs".

3.1.- Contact agar method is only applicable to flat surfaces. A contact plate (or dipslide) filled with a suitable agar medium is pressed against the surface to be tested. After incubation, an estimate of the surface contamination is obtained by counting the number of developed colonies. This sampling method is not applicable to pathogen detection methods, when a pre-enrichment step is required.

Contact plates, also named RODAC (Replicate Organisms Direct Agar Contact) are plastic dish with diameter 65 mm, filled with a controlled volume of agar medium (chosen according to the target microorganisms), especially made for the sampling of surfaces. Dishes vary in diameter or area, according to the type of surface to be sampled (usually aprox. 25 cm^2), considering the agar shall form a convex meniscus with the dish.

Dipslides are synthetic slide (7 cm^2 to 10 cm^2), one or both sides of which are covered with a layer of a solid growth medium (chosen according to the target microorganisms).

Surfaces are analysed by pressing the agar surface of the contact plate or the dipslide firmly and without any lateral movement against the flat and regular test surface. For a reproducible and optimal results, it is recommended (ISO 18593:2004) a contact time of 10 s and a pressure obtained with a mass of 500 g. These specific conditions can be obtained using specific devices like Count-Tact© (BioMerieux ref. 96300).

Example of RODAC plate (with central grid)

RODAC plate + Count-Tact© (From BioMerieux)

Dipsilde (From 3M)

Figure 2: Direct contact agar sampling

3.2.- Swab method can be used for all types of surfaces. For the sampling of large surfaces (> 100 cm^2), sterile cloths or sponges can be used. This alternative method is useful for the estimation of the microbial load of surfaces. Using the swab method, a specified area of the surface to be examined is marked (e.g. using a template) and then wiped.

The ideal sample collection material is one that (Ward, 2012):

1. Is free of any toxic substances that would cause injury or be lethal to microorganisms after the surface sample has been collected and before the sample has been tested.
2. Is sufficiently rugged so that the surface can be vigorously scrubbed to disrupt and lift biofilm without disintegration of the collection material.
3. Is effective at collecting the microorganisms from the surface.
4. Releases all of the microorganisms during a procedure designed to count levels of microorganisms collected.
5. Does not interfere with a diagnostic test performed on the sample and produce false positive or false negative results.

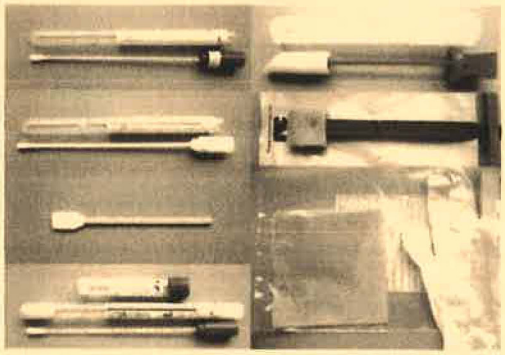

Figure 3: Different swabs and sampling devices (from Faille et al, 2014)

Polyurethane foam Sponge Samplers (eg. Ref. EZ Reach™ Worldbioproducts) is designed to allow the user to aggressively sample an environmental surface to disrupt biofilms and lift strongly attached cells. It is less likely to fragment or tear during sampling and big areas can be sampled. Bacteria can survive on the polyurethane sponges up to 72 hours at refrigerated temperatures.

Biocide-free cellulose sponges are manufactured from natural materials such as wood pulp and vegetable fibers; some batch to batch variability in chemical and/or mechanical properties may occur. Cellulose sponges have been recommended by the USDA for carcass sampling.

Dacron fibers are usually applied to small areas with limited access and may fray or unwind during sample collection. In the study conducted by Botrugno et al., 2015, flocked tipped swabs (COPAN SRK™) showed an average recovery of 69,6% more than classic dracon (viscose) swabs.

Faille et al., 2014 evaluated the interaction between swab materials versus sampling surfaces for biofilm recovery, showing there is not a single swab material giving a high recovery in all surfaces (see Table 1)

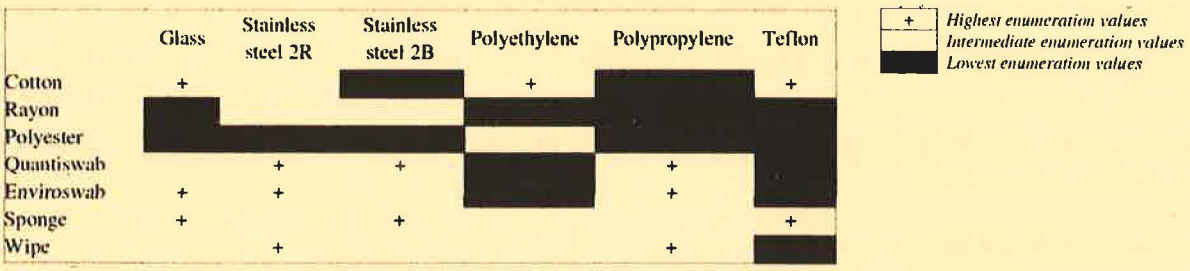

Table 1: Ability of microbial tests to detect biofilms on the different materials

Swabbing can be performed in dry conditions (usually to collect contamination in dry environments like milk powder, chocolate) or in wet conditions. Wet swabbing usually allows a better recovery in different surfaces compared with dry swabbing (see Figure 4)

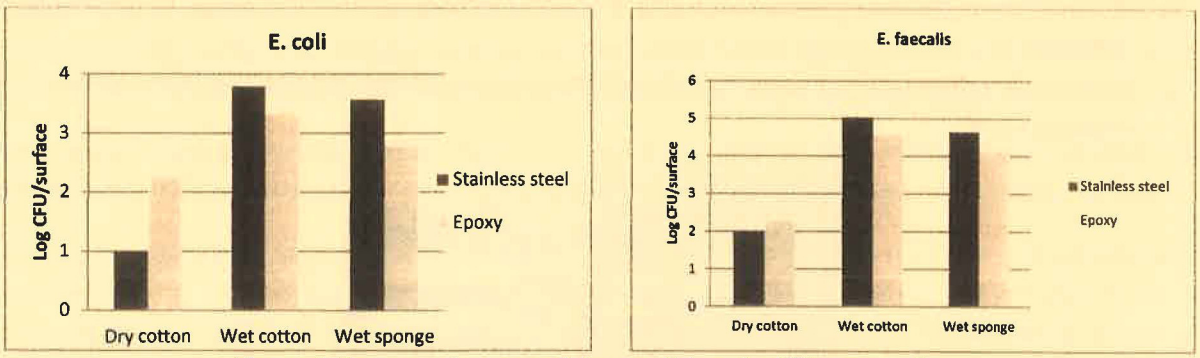

Figure 4: Recovery in wet and dry conditions in different surfaces

After swabbing, the swab sticks are broken into a tube or bottle containing a sterile dilution fluid or neutralizing fluid and mixed by hand. If the surface is wiped with a sterile (damp) cloth or sponge, the sampling device is stored in a known volume of dilution liquid (e.g. 100 ml for 100 cm^2).

In the laboratory the initial suspension and, if necessary, further decimal dilutions are used to determine the presence or the number of microorganisms using the procedures described in the methods for the enumeration or detection of the (groups of) microorganisms to be investigated.

In both cases, after sampling, the surface is cleaned and disinfected, if necessary, to avoid traces of nutrients resulting from the sampling procedure remaining on the sampled surface.

4.- Neutralizers and diluents

A collection broth has two primary purposes (Ward et al., 2012):

- To neutralize sanitizers that may be present on the surface that is being sampled.
- To maintain the viability of the microorganisms after a sample is collected and until the sample is processed by the laboratory.

In general, the base for neutralizing liquid is buffered peptone water, or peptone salt, or any other appropriate diluent (such as quarter-strength Ringer's solution, phosphate buffer at pH 7,5, peptone solution at 1 g/l). However other diluents have been developed to improve transport and viability of bacteria (see Table 3).

In cases where residues of disinfectants are expected, appropriate neutralizers should be added to the dilution fluid and to the media used on the contact plates to prevent any inhibitory effect of the disinfectants on the growth of microorganisms. Disinfectants are generally formulated for a disinfection contact time of 5 min to 15 min. Wait for a period in accordance with the disinfectant specification before investigating the surface with swabs or contact plates to assess the performance of the cleaning and disinfection program.

An appropriate neutralizer for all situations cannot be prescribed. A number of disinfectant neutralizers are recommended in EN 1276, EN 1650, EN 13697 and EN 13704. Also specific neutralizers have been developed allowing better recovery and also less interference with further steps for some specific methods (e.g. HiCap™ Neutralizing Broth for 3M petrifilm or Real Time PCR).

The components of a neutralizer which may be used in most situations can be prepared with a solution of peptone (1 g/l), sodium chloride (8,5 g/l), and the components included in the table 2.

Component	Conc.
Sorbitan monooleate (Polysorbate 80)	30 g/l
Lecithin	3 g/l
Sodium thiosulfate	5 g/l
L-Histidine	1 g/l
Saponin	30 g/l

Table 2: Neutralizer which can be used in most situations (from ISO 18593:2004)

Transport conditions can have a big influence in the final results and needs to be considered as a critical factor. Temperature, time and transport diluent combination can have a big impact on microorganisms stability during transport (See fig 5)

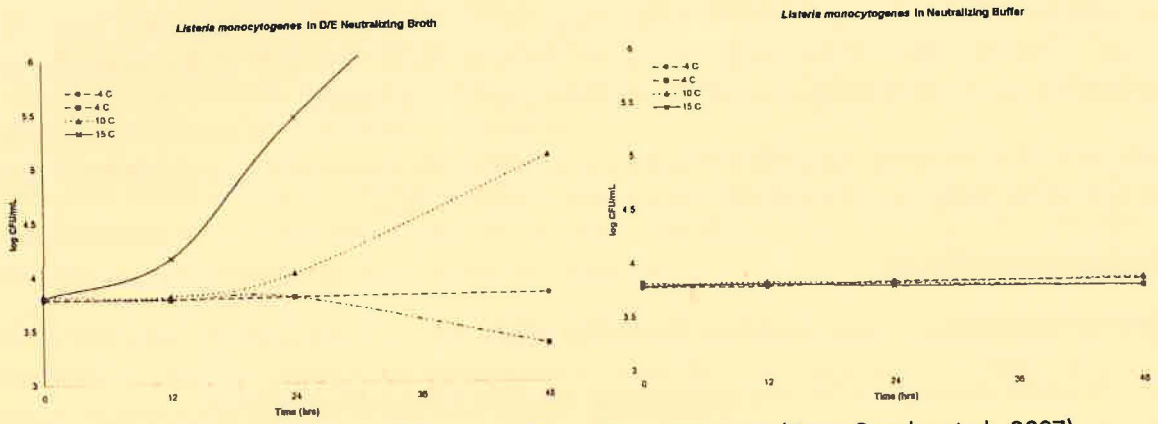

Figure 5: Listeria growth in different buffer and temperatures (From Bazako et al., 2007)

According 18593:2004, samples should be transported preferably within 4 h at 1 °C to 4 °C, in a way that no contamination can occur. The analysis must be started as soon as possible and not later than 24 h.

Despite of that, commercial information provided by suppliers shows a broad range of transport times for combinations swabs/sponge materials of neutralizers. For most of the combinations, transport times up to 48-72h at refrigeration temperature are not affecting the viability of microorganisms for further analysis. For ambient temperature transport, in most of the cases there is not information available about the impact on microorganisms but, some suppliers recommend not to exceed 48-72 hours after sampling

5.- Laboratory analysis

Contact agar plates must be incubated according to the type of microorganisms to be enumerated (remember this sampling is not applicable to qualitative methods).

For swabs in tubes with neutralizing/transport diluent, thoroughly mix the contents of tubes containing swabs using a mixer for 30 s, adjusting the speed so that the wall of the tube is wetted up to a height of 2 cm to 3 cm below the top. This step is critical to avoid the collected microorganisms don't remain trapped in the tip of the swab instead of being easily released (Ismaïl et al., 2013). Enough diluent/neutralizing liquid must be used to guarantee the entire sample is immersed (e.g. for big samples like shocks, cloths or sponges).

Swabs + diluents/neutralizing liquids should be considered the initial suspension. For qualitative methods, the initial suspension must be incubated for pre-enrichment according the instructions described in the specific method.

For enumeration, an aliquot from the initial suspension must be plated. If high numbers of microorganisms are expected, prepare further decimal dilutions and proceed with the enumeration method.

Swabs can be also analysed by ATP measurement, allowing a rapid result (in minutes). However, this techniques are not targeting only microbiological contamination but all organic material present in the surface, giving a total result in Relative Luminiscence Units (RLU)

6.- Calculation of results

6.1.- Contact agar methods, results can be estimated easily by counting the total number of colonies in the surface of the agar. For an accurate enumeration, the effective surface of contact agar should be considered. For example, if the agar surface is Ø 55 mm, this corresponds to a surface of 23.75 cm^2.

Also it is possible to count (and report) only the colonies present in the central grid, knowing each square is equal to 1 cm^2 (total surface = 16 cm^2).

6.2.- "Swabs" methods, the number of colonies per surface (CFU/cm^2) of surface N_S using the formula:

$$Ns = \frac{N \times F}{A} \times \frac{1}{d}$$

where

- N: is the number of CFU in a plate (considering 1 mL of diluent has been poured);
- F: is the amount, in mililitres, of diluent in the tube or homogenizer bag;
- A: is the surface investigated, in square centimetres;
- d: is the dilution tested.

If the area swabbed was not defined, calculate the CFU/sample N_{sw} using the formula:

$$Nsw = N \times F \times \frac{1}{d}$$

Example:

A surface of 100 cm^2 has been swabbed. The swab was diluted in 5 ml of BPW (initial suspension or 10^{-1}). 1 mL from the initial suspension was plated in a Petri dish. After incubation, 12 colonies were detected in the plate.

The final count in CFU/cm^2 is:

$$Ns = \frac{N \times F}{A} \times D = \frac{12 \times 5}{100} \times \frac{1}{10^{-1}} = 6 \text{ CFU/cm}^2$$

In case of qualitative methods when an enrichment step is included, the target microorganism must be reported as detected or not detected in the area swabbed, or per sample if the area is not known.

Results for environmental sampling are often presented as hygiene scores based on the presence of a pathogen or a number of colony-forming units (CFU) per sample or square centimeter present on a test surface. Usually these methods are not enough not quantitatively reliable or reproducible and results should only be used in a "trend analysis". Limits of control should be stablished for each specific use.

7.- New technologies

Some new technologies have been developed to improve the environmental sampling in food industries. These methods are mainly focusing on Listeria detection in surfaces.

The prototype developed in the project BioliSME, supported by the European Commission within the Seventh Framework Programme, (FP7-SME-2011-286713) was able to improve the recovery from Listeria monocytogenes biofilm on stainless steel and polytetrafluoroethylene surfaces from 11% by conventional techniques (swabbing) to a 98 % by using a device based on air/water ablation (Gião et al., 2015). This device combined with a detection system based on biosensor technology allowed the detection of 10^3 cfu of Listeria monocytogenes in 10 minutes.

Other commercial method available is the Sample6 DETECT/L™ Test developed in USA (www.sample6.com). The method allows the detection of Listeria spp. in 6 hours in stainless steel with a Probability Of Detection (POD) equivalent to the reference method (POD < 1). The detection technology applies next-generation synthetic-biology techniques (Lu et al., 2013) to enable bacterial pathogen detection from a swab or sponge. The system uses the inherent specificity of naturally occurring bacteriophage that, leads to the production of the light-producing enzyme luciferase. Since production of luciferase is an active biological process, Detect/L™ can detect only living cells.

In both cases further validation studies against the reference method will be needed to allow a full implementation and recognition by food companies and regulatory authorities.

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