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Research Letter

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European survey and evaluation of sampling methods recommended by the standard EN ISO 18593 for the detection of *Listeria monocytogenes* and *Pseudomonas fluorescens* on industrial surfaces

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One sentence summary: This article assesses for the first time the effectiveness of sampling methods recommended by the standard EN ISO 18593.

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ABSTRACT

The ready-to-eat products can be contaminated during processing by pathogen or spoilage bacteria, which persist in the industrial environment. Some bacterial species are able to form biofilms which protect them from environmental conditions. To check the bacterial contamination of the surfaces in the food industries, the professionals must regularly use

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surface sampling methods to detect the pathogen such as *Listeria monocytogenes* or the spoilage such as *Pseudomonas fluorescens*. In 2010, we designed and carried out a European survey to collect surface sampling information to detect or enumerate *L. monocytogenes* in food processing plants. A total of 137 questionnaires from 14 European Union Member States were returned. The outcome of this survey showed that the professionals preferred friction sampling methods with gauze pad, swab and sponges versus contact sampling methods. After this survey, we compared the effectiveness of these three friction sampling methods and the contact plates, as recommended in the standard EN ISO 18593 that was revised in 2018, on the recovery of *L. monocytogenes* and of *P. fluorescens* in mono-specie biofilms. This study showed no significant difference between the effectiveness of the four sampling methods to detach the viable and culturable bacterial population of theses mono-specie biofilms.

Keywords: Standard EN ISO 18593; sampling methods; food industry; biofilm; food contact surfaces

INTRODUCTION

Bacteria are able to adhere to surfaces in industrial environments and can develop in favorable conditions to form a biofilm. A biofilm consists a complex consortium of microorganisms embedded in an extracellular matrix (López, Vlamakis and Kolter 2010). Food-processing environments provide a variety of conditions, which might favor the formation of biofilm (presence of moisture, nutrients and microorganisms from the raw food or the environment (air, ….). Such biofilms are potential sources of food cross-contamination (industrial surfaces from food) in industries that may lead to spoilage or transmission of food-borne pathogens (Gunduz and Tuncel 2006). Moreover, when a part of biofilm detaches from an abiotic surface, bacterial cells can easily disseminate and colonize other locations in the processing plant (Mkhungo, Oyedeji and Ijabadeniyi 2018).

Cleaning and disinfection (C&D) operations, that can be carry out simultaneously or successively one after the other, are among the prerequisites to provide a good hygienic food environment. C&D operations must be precisely described in the company's sanitary control plan and the performance and effectiveness of these operations must be controlled (article 5.2 of EC Regulation 2073/2005). To achieve its hygienic controls, the professionals use sampling methods described in the revised standard EN ISO 18593:2018 or the European Guidelines (Carpentier and Barre 2012).

Different sampling methods can be used to sample material surfaces in food processing plants. There were two categories of sampling methods: friction (swab, gauze pad and sponge) and contact (contact plate, contact blade and petrifilms™). Unfortunately, no large survey is available in the literature regarding the sampling practices in food industries. This standard aims to specify horizontal methods for sampling methods using contact plates, stick swabs, sponges and gauze pad on surfaces in the food chain environment in order to detect and enumerate culturable microorganisms such as pathogenic or non-pathogenic bacteria or yeasts and molds. However, in this standard, no information are mentioned on the relative effectiveness of the different sampling methods. In the literature, some studies reported that sampling methods by friction are generally considered to be more sensitive than direct sampling using fingerprint methods (Gomez *et al.* 2012; Luyckx *et al.* 2015). There are no data available so far to allow professionals from the food sector to compare the relative effectiveness of different sampling methods for the quantification of residual bacteria on material surfaces.

The first step of this study was a survey to determinate the sampling methods used by the European food companies to detect or enumerate bacteria *Listeria monocytogenes* on material surfaces (food contact and non-food contact surfaces). In a second step, we evaluated the effectiveness of the four sampling methods most commonly used by food business operators (FBOs) and described in the standard EN ISO 18593:2018 to detect *L. monocytogenes* and *Pseudomonas fluorescens* in a mono-specie biofilms.

MATERIAL AND METHODS

European survey on sampling methods

This survey was designed to collect information about FBOs' sampling practices on food-contact and non-food contact surfaces to detect or enumerate *L. monocytogenes* in food processing plants. The European Union Reference Laboratory for *L. monocytogenes* (EURL *Lm*) prepared and sent a questionnaire to the network of National Reference Laboratories (NRLs) for *L. monocytogenes* in order to get a state-of-the-art overview of the sampling methods used to detect *L. monocytogenes* in food processing plants.

A questionnaire about 'sampling *L. monocytogenes* from processing plants' was sent in June 2010 to each of the 34 NRLs of the EURL *L. monocytogenes* network.

Each NRL was in charge of forwarding the questionnaire to national FBOs, to national service providers and to official control services. This questionnaire covered different aspects of sampling procedures applied in food processing plants for the detection of *L. monocytogenes* on surfaces.

Bacterial strains and growth conditions

Two species commonly isolated in food processing plants were studied: *L. monocytogenes* (serogroup 1/2a-3a) strain Lm1 and *P. fluorescens* strain 4C29 isolated from industrial surface samples in seafood processing plants and were stored in brain–heart infusion medium (AES, Combourg, France) supplemented with glycerol (18% v/v) at −80°C in cryotubes. Before each experiment, 1 ml of culture from a cryotube was transferred into 9 mL of tryptone soy broth (TSB, Oxoid, Dardilly, France) and incubated for 24 h at optimal temperature (37°C for *L. monocytogenes* and 30°C for *P. fluorescens*). One milliliter of suspension was transferred to 9 mL of TSB and further incubated for 8 h at the same temperature. One milliliter of suspension was transferred to 100 mL TSB and incubated overnight at the same optimal temperature. Cultures were centrifuged at 5000 g for 10 min and the pellet was washed with 100 mL of sterilized saline water. The procedure was repeated twice and the final pellet was re-suspended in 2.5 mL TSB diluted 1/10 in sterilized saline water. Inoculum concentrations of *L. monocytogenes* and of *P. fluorescens* were adjusted to 10^7 CFU mL^{-1} in sterilized saline water.

Biofilm formation

Coupons of AISI 316 L 2B stainless steel (Sapim Inox, Loison-sous-Lens, France) of 37 mm × 16 mm and polyurethane (PU) of 40 mm × 20 mm (Ammeraal Beltech, Noyelles-lès-Seclin,

France) were washed as described in (Midelet and Carpentier 2002) and were disinfected with a P3-Topactive OKTO solution (Ecolab, Saint-Paul, Minnesota, USA) diluted at 0.3% (v/v) for 5 min. Coupons were then rinsed with sterilized distilled water and placed horizontally in 67 mm diameter Petri dishes. Ten millilitres of the bacterial suspension of *L. monocytogenes* and of *P. fluorescens* adjusted to 10^7 CFU mL^{-1} in sterilized saline water were deposited in each Petri dish, which were incubated at 20°C or 8°C for 48 h. After incubation, non-adherent bacteria were eliminated by two successive soakings in two vials containing 100 mL of sterilized saline water and coupons were subsequently placed in new sterile Petri dishes.

Sampling methods tested

Four sampling methods were tested.

Stick-mounted sponges supplied in sterile bags with 10 mL of buffered peptone water broth without neutralizer (Sponge-Stick, 3M, Minnesota, USA) were used as recommended by the standard NF EN ISO 18593 i.e. by applying them in two perpendicular directions, changing the face of the sponge and making sure to cover the entire area. After sampling the surfaces, sponges were treated in the lab blender (Stomacher, VWR, Fontenay-sous-Bois, France) for 1 min. Supernatants were kept for quantification analyses.

Gauze pads were supplied with 10 mL buffered peptone water broth without neutralizer (Humeau laboratory, La Chapelle-sur-Erdre, France). Before use, gauze pads were cut into pieces of 4 × 4 cm^2. Sampling was performed as recommended by the supplier. Piece of gauze pad was then placed in Stomacher bag containing 8 mL of buffered peptone water broth and homogenized for 1 min in a lab blender. Supernatants were kept for subsequent analyses.

The recovery by swabbing was performed with one cotton swab supplied in sterile tubes (VWR, Fontenay-sous-Bois, France). Biofilms were swabbed with one swab wetted with buffered peptone water broth and applied as recommended by the standard NF EN ISO 18593 i.e. in two perpendicular directions, changing the face of the swab and making sure to cover the entire area. The swabs were submerged in a microtube containing 2.5 mL of sterilized saline water. The microtube containing broken swabs was vortexed for 20 s. Supernatants were then used for further enumeration analyses.

In addition to these three methods, direct contact plates were also tested as recommended in the EN ISO 18593 standard. Contact plates without neutralizer (Count-Tact® Agar, BioMérieux, Marcy-l'Étoile, France) were applied on the contaminated coupons with an applicator (Humeau laboratory, La Chapelle-sur-Erdre, France) for 10 s at a pressure of 500 g (according to standard NF EN ISO 18593). To quantify bacteria, agar was removed from the plate with a sterile spatula and placed in a sterile container previously filled with 10 mL of sterilized saline water. The suspension was vortexed for 20 s and supernatants were kept for quantification analyses.

Viable culturable bacterial enumeration

Supernatants obtained with each sampling method were diluted with sterilized saline water, plated on ALOA® agar (BioMérieux, Craponne, France) and counted after incubation at 37°C for 24 h as stated in standard EN ISO 11290-2 for *L. monocytogenes*. *Pseudomonas fluorescens* was enumerated from each supernatant using tryptone soy agar (TSA, Oxoid, Dardilly, France) plates incubated for 24–48 h at 30°C.

Statistical analysis of the data

All the experiments were performed in 16 replicates for each bacterial species and each sampling methods. The data were analyzed for comparative analysis of efficiency of the tested sampling methods. Data were analyzed with the Statgraphics centurion XVI software (Sigma plus, Paris, France). Paired Student t-tests were used to compare the sampling bacterial population for each sampling method and for each bacterial species. Distribution of bacterial populations was described with box and whisker plots. Differences were considered significant with $P < 0.05$.

RESULTS

Survey on the sampling methods used to detect *L. monocytogenes* from food processing plant in Europe

A total of 137 questionnaires of FBOs returned from 14 member states were analyzed. The returns from each country were as follows: 58 from France, 29 from Ireland, 17 from Germany, 7 from Finland, 5 from Portugal, 4 from Estonia, 4 from Belgium, 4 from Slovakia, 3 from the United Kingdom, 2 from Spain, 1 from Denmark, 1 from Poland, 1 from Norway and 1 from Greece. The survey respondents were representatives from all food sectors (35% multisector, 25% meat and processed meat, 16% dairy, 9% fresh fruit or vegetable, 7% poultry, 6% ready to eat meals, 1% fish and 1% chocolate).

In this survey, 55% of FBOs performed the detection of *Listeria* in their plants mainly with frequencies of daily, bi-weekly or weekly. They mainly performed sampling just after CαD operations and/or before starting a new production cycle. FBOs used preferentially gauze pad (29%), wet swab (20%), sponge (16%), brush (16%) and dry swab (7%) (Fig. 1). We noted that only 27% of the respondents used the swabs to detect *L. monocytogenes* on different material surfaces. Several FBOs (18%) reported using several types of sampling methods.

Comparison of the effectiveness of four sampling methods on the recovery of the bacteria (*L. monocytogenes* and *P. fluorescens*) on the material surfaces

After the analysis of the results of the European survey, we selected mainly three sampling methods used in food plants to evaluate their effectiveness for recovering pathogenic and spoilage bacterial biofilms: gauze pads, wet swabs and sponges. In a first step, we observed the adherent bacteria on the coupons (stainless steel and PU) by epifluorescence microscopic after staining by a 5 μg mL−1 solution of 4', 6 diamidino-2-phenylindole (DAPI) (Fig. S1, Supporting Information). These observations confirmed the presence of *L. monocytogenes* and *P. fluorescens* biofilms on the coupons. We showed a heterogeneous structure, with the presence of some thick clusters surrounded by diffuse material and separated by slightly contaminated areas, where only small clusters and single cells could be observed. After biofilm formation, each sampling method was applied on the contaminated material surface. Bacteria were then enumerated on agar media (Fig. 2).

Regarding the *L. monocytogenes* biofilms cultivated on stainless steel (Fig. 2A), we noted that the enumeration median values were around 7.20 and 6.00 Log CFU/cm^2 at 20°C and 8°C, respectively, for all sampling methods. The population distribution was more important when the sampling methods were applied on *L.*

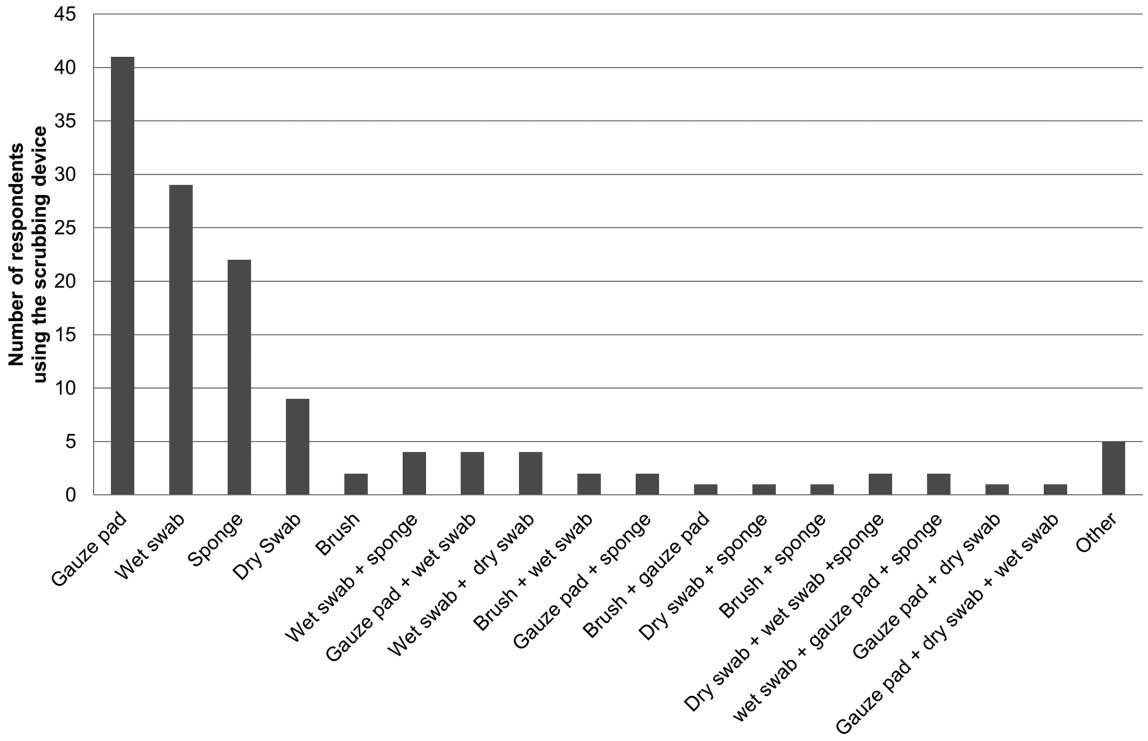

Figure 1. European survey on sampling methods used in 137 food industries in 14 different countries.

monocytogenes biofilms cultivated at 20°C compared to 8°C. The statistical analyses showed that there was no significant difference of effectiveness whatever the sampling methods used to recover *L. monocytogenes* on stainless steel at 20°C or at 8°C. For *L. monocytogenes* biofilms cultivated on PU, there was no significant difference between the bacterial recovery of the sampling methods used ($P > 0.05$) (Fig. 2B).

For *P. fluorescens*, the median values ranged from 6.45 to 6.90 Log CFU/cm^2 on stainless steel and from 6.25 to 6.80 Log CFU/cm^2 on PU for the biofilms cultivated at 20°C and 8°C, respectively (Fig. 2C and D). In all conditions tested, similar bacterial population distributions were observed. Statistical analyses did not showed significant difference on the effectiveness of the sampling methods tested.

DISCUSSION

Material surfaces of food processing environment are often contaminated with biofilms harboring pathogens such as *L. monocytogenes* or spoilage bacteria such as *P. fluorescens*. These bacteria are particularly known to persist in adverse environments, e.g. high NaCl concentrations, strong desiccation or low temperatures, which further increases their potential for cross-contamination during food processing (Hansen and Vogel 2011).

It is essential for professionals to check the effectiveness of C&D operations to eliminate bacterial surface contaminations and consequently to have effective sampling methods. In this study, the performance of the sampling methods most commonly used in food processing plants was evaluated. However, to our knowledge, no published study reported the sampling methods actually used in food companies in Europe. In first step, a European survey about the sampling methods used to detect *L. monocytogenes* in food processing plants was conducted, describing FBOs sampling practices for the detection and recovery of *L. monocytogenes*. A total of 137 questionnaires from 14 countries were returned. This survey was representative of the sampling methods used in all food sectors in Europe and showed that all the professionals preferred friction sampling methods over contact sampling methods for the detection of *L. monocytogenes*. Among these friction sampling methods, a great diversity of practices of sampling methods was observed. Indeed, several respondents declared inadequate practices according to the EN ISO 18593 standard (data not shown). For example, one of the main reasons concerned the area to be sampled. It is recommended in the EN ISO 18593 standard that the total sampled area during a sampling campaign should be as large as possible to increase the probability to detect *L. monocytogenes*. In this regard, it is advised to sample between 1000 cm^2 and 3000 cm^2 (i.e. 0.1 m^2 to 0.3 m^2) when possible. The survey also revealed the use of alternative sampling methods included in the 'others' category of the survey. For example, some FBOs used sampling methods by non-woven microfiber towel, wet towel or hygienic tampon. Several FBOs indicated that they used a combined surface sampling methods (e.g. gauze pad + dry swab). Indeed, they used multiple sampling methods in their food processing plant with a same sampling method for a specific area. The sampling methods were systematically applied individually. That is why we have applied them individually in this study.

We tested the sampling methods most commonly used by FBOs and recommended by the standard EN ISO 18593. We did not select the brush because the survey showed that few FBOS used this technique. Furthermore, the brush was no longer marketed and there was no standard protocol for this technique. We demonstrated that *L. monocytogenes* and *P. fluorescens* were able to form single species biofilms in conditions tested that were encountered in food processing environments on different surfaces (stainless steel and polyurethane) at 8°C to mimic industrial conditions and at 20°C to mimic a optimal temperature for the best growth conditions.

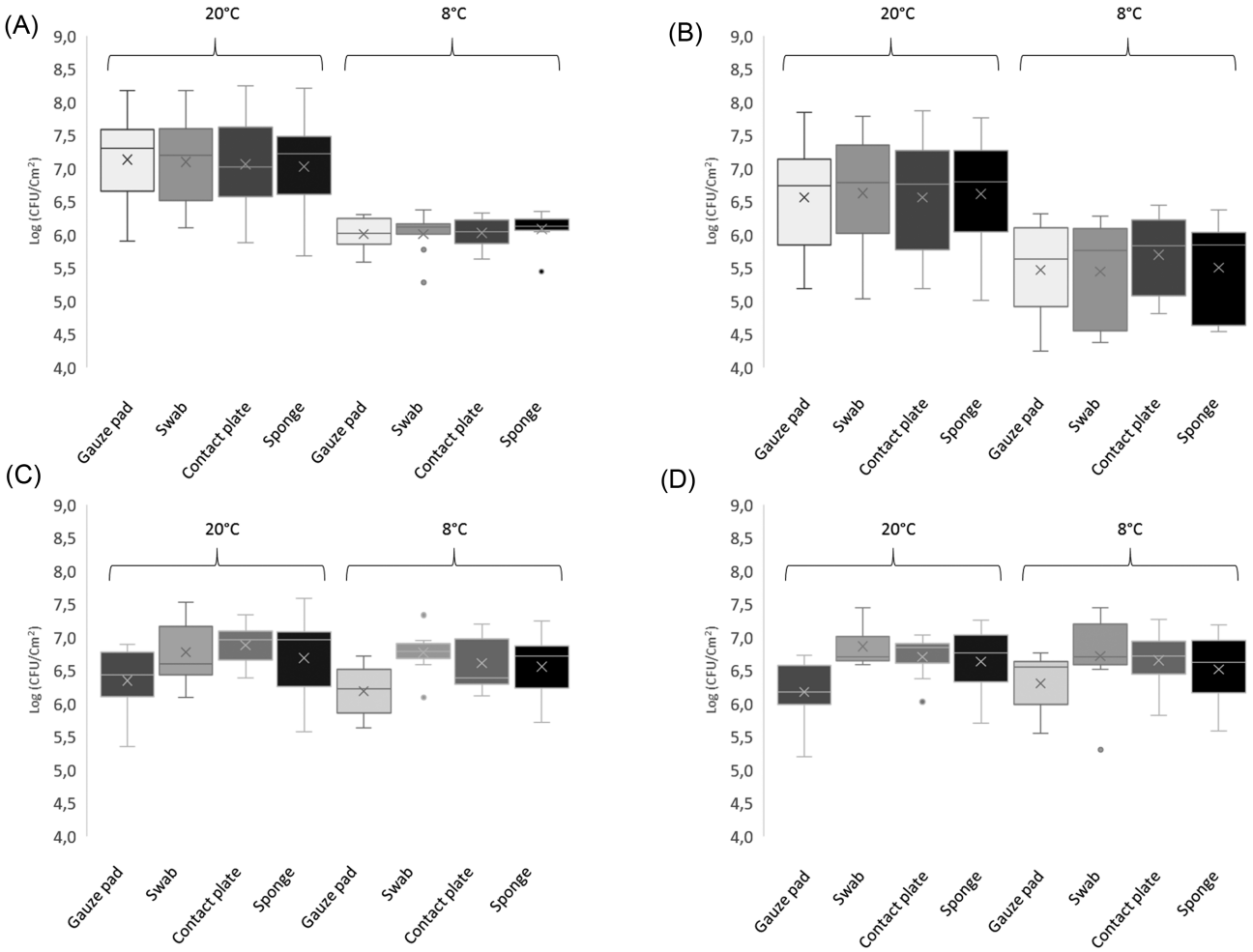

Figure 2. Comparison of sampling methods by gauze pad, swab, contact plate and sponge on: **(A)** *L. monocytogenes* biofilms cultivated on stainless steel at 20°C and 8°C, **(B)** *L. monocytogenes* biofilms cultivated on polyurethane at 20°C and 8°, **(C)** *P. fluorescens* biofilms cultivated for 48 h on stainless steel at 20°C and 8°C, **(D)** *P. fluorescens* biofilms cultivated for 48 h on polyurethane at 20°C and 8°C. The grey lines indicate the median, the boxes are limited by the first and third quartiles Q1 and Q3 (25 and 75% of data below these values, respectively), the whiskers contain values between Q1–1.5 (Q3–Q1) and Q3–1.5 (Q3–Q1), and outer values appear as individual dots.

In our study, no significant impact of the surface type was observed regarding the amount of detached bacteria, despite substantial differences in the topography and hydrophobic properties of the materials (data not shown). Indeed, only very small amounts of residual biofilms were observed by microscopy on both surfaces (data not shown). These results are not consistent with those previously reported, thus suggesting for example that the sampling efficiency of cellulose sponges (Krauter *et al.* 2012) or nylon-flocked-swabs (Probst *et al.* 2010) was affected by the surface roughness. This inconsistency might be explained by the shape of the large size of the surface irregularities, which in our study did not protect adherent bacteria from shear forces during surface friction. The two culture temperatures tested did not significantly impacted the effectiveness of sampling methods. No similar studies are currently available in the scientific literature.

Moreover, we showed that there was no significant difference between the sampling methods to detach the viable and culturable *L. monocytogenes* or *P. fluorescens* populations from two material surfaces (stainless steel or PU) at 8°C or 20°C. To our knowledge, this is the first study comparing the bacterial detachment of various sampling methods (contact plates, sponges, gauze pads and swabs) for one Gram+ and one Gram− bacteria in industrial environment conditions. Gomez *et al.* (2012) studied the effectiveness of nine sampling procedures for the recovery of *L. monocytogenes* from a stainless steel table inoculated with 10^5 CFU/100 cm^2. Sponge (premoistened cellulose), cotton swab and gauze pad (cotton) exhibited lower recovery rates than contact plates i.e. 0.97%, 0.09% and 0.35%, respectively. Based on an analysis of 240 samples collected in the meat processing environment, Kovacevic *et al.* (2009) showed that the cotton swab method was significantly ($P < 0.01$) less efficient in recovery of *Listeria* spp. than the sterile-sponge and composite-ply tissue methods. These differences in results can be explained by several factors: impact of various surface conditioning (by organic materials) and nature of the sampled surface (polarity and roughness of the material). There is an open question regarding the potential results if the four sampling methods could have been tested under industrial conditions, as the biofilms produced under lab conditions could have been relatively easy to detach. Indeed, biofilms may develop differently in the processing plant and be potentially more resistant to stalling by sampling methods. For example, they may develop under dynamic conditions or be sub-

jected to different stresses (biocidal, hydric and oxidative) that would make them more adherent. The development of biofilms in companies could also be more important because of the stages of famine or the association of several bacterial species. However, a comparative study in a processing plant is difficult since the heterogeneity of surface contaminations is very important.

In conclusion, the present study showed that most of the professionals from the food industry preferred friction sampling methods over contact sampling methods for the detection of *L. monocytogenes*. It also allowed us to show that among these friction sampling methods, there is a great diversity of practices for surface sampling, ranked in order of use as follows: gauze pad, swab and sponges. Our comparative study is on the effectiveness of these three sampling methods and the contact plate method that are recommended in the standard EN ISO 18593 for detecting and quantifying *L. monocytogenes* and *P. fluorescens*. We showed, in lab conditions, that the four sampling methods tested had permitted to detach similar levels of bacteria from two material surfaces used mainly in the food processing environment. This tends to show (even though further investigations should be conducted) that FBOs should adapt the choice of their sampling methods on the basis of the area to be sampled (large areas vs small low accessibility areas or smooth vs rough surfaces) rather on their assumed recovery effectiveness.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interests. None declared.

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