WILEY EFSA JOURNAL

Urgent scientific and technical assistance to provide recommendations for sampling and testing in the processing plants of frozen vegetables aiming at detecting Listeria monocytogenes

Journal:	*EFSA Journal*
Manuscript ID	EFSA-2018-0141
Wiley - Manuscript type:	Supporting publication
Date Submitted by the Author:	29-Jun-2018
Complete List of Authors:	Author, EFSA; European Food Safety Authority, COMMS
EFSA Question Number:	EFSA-Q-2018-00347
Keywords:	L. monocytogenes, vegetables, fruits, freezing plant, handling facilities, critical sampling site, microbial source tracking
Abstract:	

TECHNICAL REPORT

APPROVED: 29 June 2018

doi:10.2903/sp.efsa.2018.EN-1445

Urgent scientific and technical assistance to provide recommendations for sampling and testing in the processing plants of frozen vegetables aiming at detecting *Listeria monocytogenes*

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Abstract

EFSA was requested to provide recommendations to the European Commission on the sampling strategies and established microbiological methods most appropriate for maximising the sensitivity of detection of *L. monocytogenes* in processing water and the environment of premises producing frozen fruits, vegetables or herbs (FVH) as well as on the final food produced; and on the identification of critical sampling sites (CSSs) for environmental monitoring of *L. monocytogenes*. Seven steps are defined for a fit-for-purpose sampling strategy which is expected to support competent authorities and food business operators in foodborne outbreak investigations where frozen FVH are implicated. The relevant CSSs can be defined based upon critical inspection inside a freezing plant and the background information described in this report. Typical non-food contact surfaces where *L. monocytogenes* can harbour in a freezing plant include: floors, especially cracks and crevices, walls, drains, ceilings, overhead structures, catwalks, wash areas, condensate and standing water, wet insulation in walls and around pipes and cooling units, rubber seals around doors, especially in coolers, metal joints, specially welds and bolts and contents of vacuum cleaners. *L. monocytogenes* is also commonly found on equipment used for food processing, preparation, storage, and transportation such as freezing tunnels, castings bowls, blade spinner, slicers, knives, cutting boards, conveyor belts, gloves joints, gaskets and dead ends. A concerted effort should be made to plan the sampling around production batches and environmental CSSs. Sampling procedures should be performed as exhaustively as possible covering the largest number of CSSs and samples per CSS to gain insight into the potential variability of the contamination sources. EN ISO standard method 11290-1 is recommended for *L. monocytogenes* detection. Characterization of *L. monocytogenes* isolates using well established molecular techniques is needed to identify strains from positive samples and establish links between isolates from humans and from implicated FVH.

Keywords: *L. monocytogenes,* vegetables, fruits, freezing plant, handling facilities, critical sampling site, microbial source tracking

Requestor: European Commission

Question number: EFSA-Q-2018-00347

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Suggested citation: EFSA (European Food Safety Authority), Allende A, Barre L, Jacxsens L, Liebana E, Messens W, Sarno E and da Silva Felicio MT, 2018. Urgent scientific and technical assistance to provide recommendations for sampling and testing in the processing plants of frozen vegetables aiming at detecting *Listeria monocytogenes*. EFSA supporting publication 2018:EN-1445. 41 pp. doi:10.2903/sp.efsa.2018.EN-1445

ISSN: 2397-8325

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

In November 2017, Finland launched an Urgent Inquiry (UI-444) in EPIS FWD describing a cluster of *Listeria monocytogenes* serogroup IVb, sequence type (ST) 6, confirmed by Whole Genome Sequencing, with cases detected in different parts of Finland since October 2016. Four Member States reported human cases associated with the same outbreak reported by Finland.

In January 2018, frozen corn was identified to be the possible source of the outbreak. Despite withdrawal and recall of specific frozen corn batches conducted in some Member States, cases continue to be reported and the outbreak does not appear to slow down. Instead it seems to increase in its intensity and peaking in recent weeks. Since the Joint ECDC-EFSA Rapid Outbreak Assessment published on 22 March 2018[1], 10 new outbreak cases have been reported. As of 18 April 2018, this outbreak has been associated with 42 cases and six deaths identified. Until the source of contamination has been appropriately controlled, there is a risk of having additional cases associated with this outbreak.

L. monocytogenes is ubiquitous in the environment (e.g. moist environments, soil, and decaying vegetation). This pathogen can persist in the food processing environment and could pose a greater risk of cross-contamination of different food productions lines. One of the mechanisms that facilitate such persistence is biofilm formation.

In view of the above, and further to discussions between our colleagues, EFSA is requested, in the framework of Article 31 of Regulation 178/2002[2], to provide scientific and technical assistance for the design of sampling and testing strategies for the detection of *L. monocytogenes* in the processing plants of frozen vegetables. This is expected to support competent authorities and food business operators in the ongoing multi-country outbreak investigation. In particular, EFSA is asked to provide recommendations on:

1. the sampling strategies and established microbiological methods most appropriate for maximising the sensitivity of detection of *L. monocytogenes* in processing water and the environment of premises producing frozen vegetables, as well as on the final food produced;
2. the identification of critical sampling sites for environmental monitoring of *L. monocytogenes* in the processing plants for frozen vegetables. This should take into account aspects related to the persistence and growth niches of *L. monocytogenes*.

1.2. Interpretation of the Terms of Reference

It was clarified with the requestor that in addition to frozen vegetables, frozen fruits and frozen herbs will also be considered in this technical report. Fruits, vegetables or herbs (FVH) that have undergone heat treatment (e.g. by cooking or drying) or any other type of processing (e.g. high pressure treatment) resulting in a shelf-stable product (such as jams, preserves and heat-treated vegetable juices) are out of the scope of this report.

The present assessment focuses on the processing environment producing frozen FVH in the European Union/European Economic Area (EU/EEA). In this report, these will be referred to as freezing plants. As frozen products may be further distributed in bulk packages to other facilities where frozen FVH can be further processed, mixed with other frozen FVH or other frozen ingredients and packaged, the environments handling these products will also be considered. In this report, these will be referred to as handling facilities. The previous stages (i.e. the pre-harvest practices and the harvesting environment) as well as the later stages (i.e. retail and catering including domestic and commercial environment) are not considered in this report.

[1] https://www.efsa.europa.eu/en/supporting/pub/1402e
[2] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

The identification of critical sampling sites (CSSs) for the environmental monitoring of *L. monocytogenes* in the processing plants of these frozen food products will consider:

- products that are blanched as well as those that are not blanched
- products undergoing size reduction operations as well as those that are not subjected to these operations; and
- the physical environment and equipment used in freezing plants and handling facilities including food and non-food contact surfaces as well as the processing water.

Sampling and monitoring can be used for different purposes within the food supply chain including: (i) product sampling for batch control/batch release, (ii) product sampling in the framework of baseline surveys, monitoring or surveillance to detect prevalence in a food product/food chain, (iii) product and/or environmental sampling in the framework of validation or verification of quality or food safety management systems (QMS/FSMS) present with food business operators (FBOs), and (iv) product and/or environment sampling for Microbial Source Tracking (MST) to identify the point of origin of a microbial contamination in a food processing environment (FPE). The sampling and monitoring recommendations described in this report have the latter purpose as the aim is to establish the sampling strategies and microbiological methods for *L. monocytogenes* detection most appropriate for maximising the sensitivity of detection of *L. monocytogenes* in the environment of premises producing frozen FVH to identify sources and routes of microbial contamination in these production sites.

2. Data and Methodologies

2.1. Data

Relevant documents were identified and reviewed by the experts in the working group. The relevant documents included EFSA scientific opinions and reports, guidance documents, ISO standards, scientific papers including review papers, books chapters, non-peer-review papers known by the experts themselves or retrieved through non-systematic searches. In addition manual searching of the reference list of these documents was performed to identify additional relevant information, as well as expert knowledge for topics where no published material is available (e.g. description of production processes).

2.2. Methodologies

Collection of information, data and scientific literature based on the knowledge and expertise of the EFSA staff and members of the Working Group drafting this scientific report were considered to answer the Terms of References (ToR(s)) of this mandate.

3. Assessment

3.1. Production of frozen fruits, vegetables or herbs (FVH)

The production processes described in this section address the current situation across EU Member States (EU MSs). As it is a complex chain process, different conditions of the production steps and activities are possible. Moreover, many frozen FVH are also imported into the EU from Third Countries, which may have been subjected to other production steps.

Frozen FVH are produced from fresh agricultural commodities. Fresh FVH are grown in a wide variety of environments depending on the commodity, the region and the growth season of the product (FAO/WHO, 2008). In most cases, FVH, after harvest, are collected in bulk containers and transported to the freezing plants. The size of the bulk container will depend on the type of commodity, as mechanical damage should be avoided. Once harvested, FVH are soon transported to the freezing plants for further processing. Intermediate storage by the farmers or intermediate trading organisations is possible only for few commodities (e.g. for hard commodities such as potatoes, apples, celeriacs) where a prolonged storage does not affect the product safety/quality when proper storage conditions are maintained. In most cases, fresh FVH are subjected to quality checks. Growers and producers are advised to establish an effective quality assurance system throughout the handling

steps between harvest and distribution, which includes the required quality checks. Safety assurance can be part of the quality assurance system and its focus is on minimizing chemical and microbial contamination during production, harvesting, and post-harvest handling of FVH. Quality control systems not only include an inspection of the quality of the fresh FVH (visual, quality properties), but might also include other type of analyses, such as analysis of pathogenic or indicator microorganisms, pesticide residues or other contaminants (e.g. nitrates or heavy metals), which are less common performed. Traceability information from the primary production settings (e.g. field sheets) is also required). Most often, there is a close collaboration between the freezing plants and their suppliers, delivering fresh FVH, for example contract production.

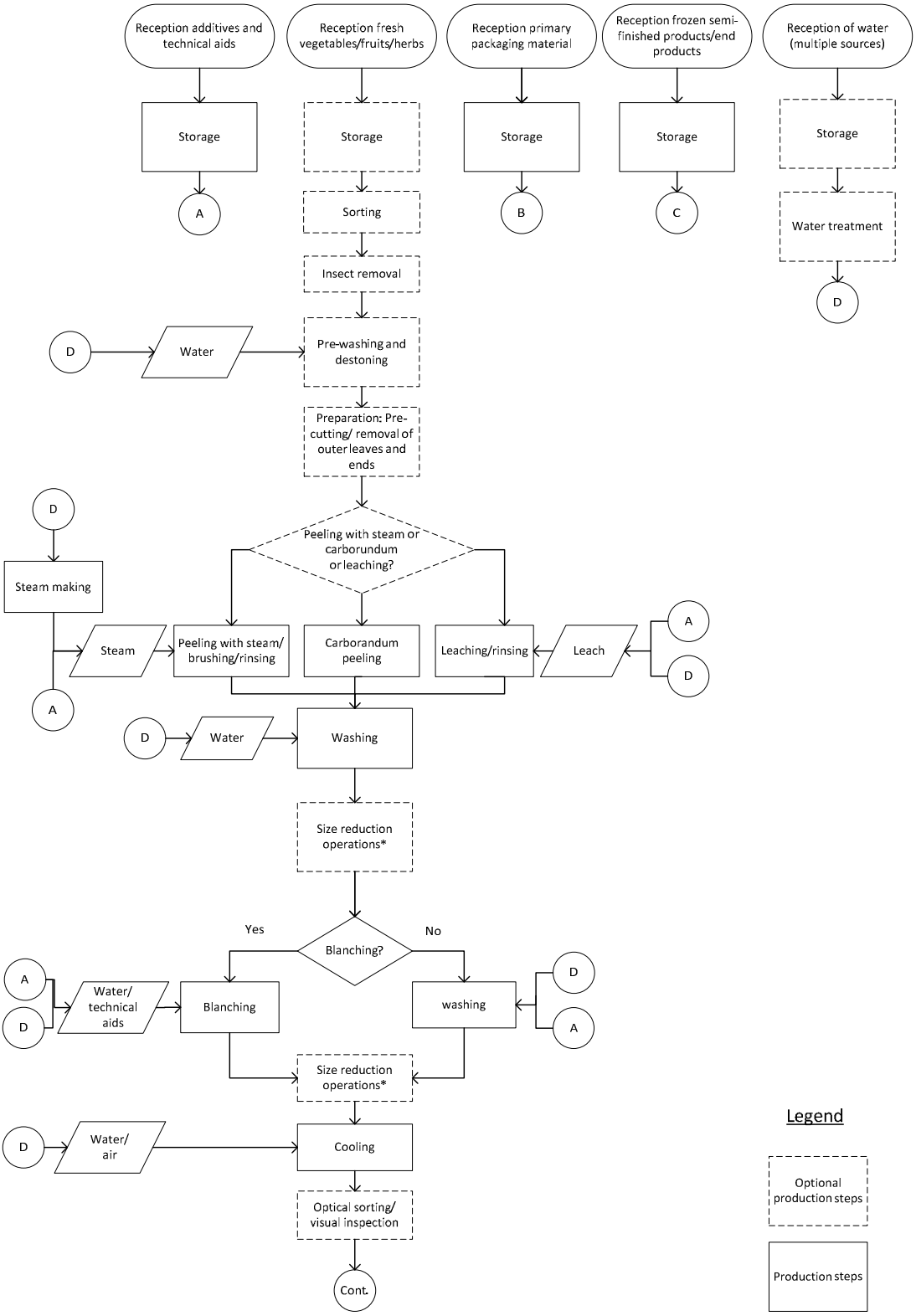
Frozen processing plants produce a wide variety of FVH. The most common frozen FVH are leafy and non-leafy vegetables (e.g. carrots, broccoli, pepper, onion, corn, and aubergine), berry fruits (e.g. raspberries, strawberries, and blueberries), pome fruits (e.g. apricots and peaches) and leafy herbs (e.g. basil, mint, chives). At the same time, frozen FVH include whole products (not cut or shredded) and those that undergo size reduction operations. They can be single products or mixed products where different FVH are present as alone or combined with other ingredients such as sauce, rice, food of animal origin (e.g. pieces of fish or meat) to get a ready-to-reheat meal. The sector is evolving towards frozen convenience food to answer the consumer demand.

A detailed description of the different production steps is given in Section 3.2.

3.2. Production steps of frozen FVH

Frati et al. (2016) have reviewed the production steps of the frozen vegetables. As previously mentioned, the frozen industry usually controls all aspects of the production chain of the frozen vegetables. FVH cultivars are selected on the basis of different quality attributes including their resistance to the mechanical stress (Kader, 1999). Time of post-harvest storage and transport to the freezing plant are kept as short as possible to avoid spoilage and fermentation of the vegetable (Frati et al., 2016). An overview of the production process is given in Figures 1 to 3 and a summary is provided in this section.

It should also be considered that, in some cases, frozen FVH that have been stored for a certain time can re-enter the production line and be re-processed. This usually occurs in the same facility as the freezing activities. However, it is also possible that bulk frozen FVH are traded towards handling facilities where frozen FVH are subjected to more simple processes which might include mixing with other frozen ingredients, packaging and storage.



* Size reduction operations can be applied at different production stages and can represent different degrees of size reduction of FVH, e.g. cutting, trimming, grating, shredding, slicing, chopping or chipping.

Figure 1: General flow chart for the production of frozen fruits, vegetables or herbs (FHV) from the moment of reception of the fresh FVH at the freezing plant (this flow chart might vary between companies and only represents a general description of the process)

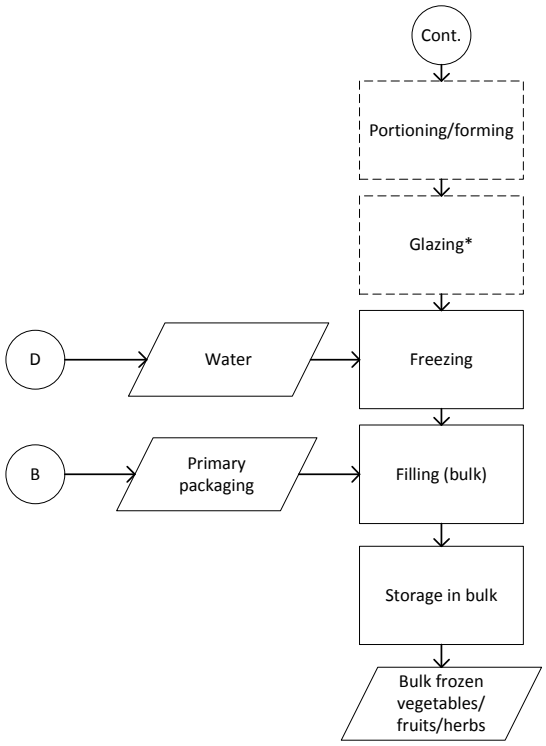


Figure 2: General flow chart for the production of fruits, vegetables or herbs (FHV) from the moment of reception of the fresh FVH at the freezing plant (continuation - this flow chart might vary between companies and only represents a general description of the process)

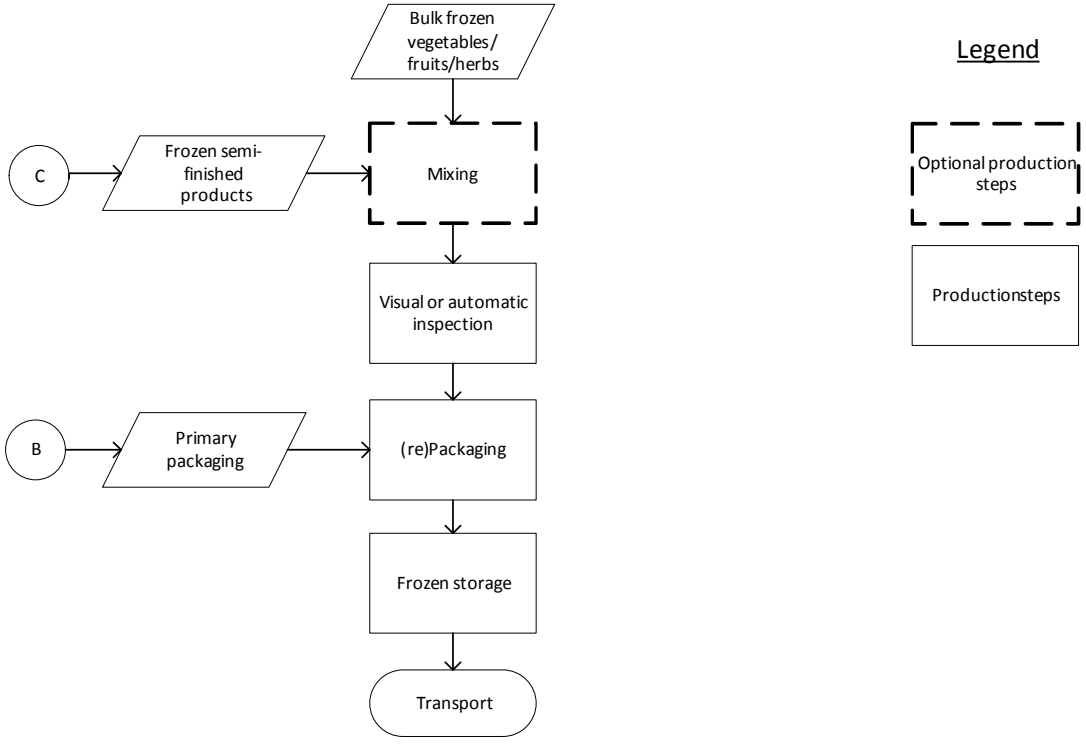


Figure 3: General flow chart of the activities conducted at handling facilities for frozen FVH (this flow chart might vary between companies and only represents a general description of the process)

3.2.1. Reception, sorting and preliminary preparation steps

Soon after harvesting, bulk fresh FVH products are transported to the freezing plant, which in the EU are mainly located near the agricultural production sites to reduce transportation time. Transportation is usually performed under ambient conditions, although refrigeration in some cases might be applied immediately after harvest and through transportation. At the freezing plant, fresh FVH are received, inspected and sorted according to the quality standards and the company's needs (e.g. size, ripeness).

The majority of fresh FVH are grown under farming contracts, which is usually translated in agreements on agricultural practices and quality standards. In this case, farmers are guided by agronomists of the processing factories on the agricultural practices they should follow (e.g. pesticide application, irrigation practices) and also the best harvest moment. In some cases, also the seeds or seedlings are provided directly by the freezing companies. Therefore, there is a close collaboration between primary producers and processors. In a minority, fresh FVH are purchased from the free market by the freezing factories.

In some cases, companies receive pre-conditioned fresh produce from different processors. This refers to fresh FVH that have been subjected to preliminary steps to eliminate the non-edible part of the produce and to adapt the size of the product to its final format (e.g. elimination of the non-edible parts, peeling, cutting) also defined as size reduction operations. These pre-conditioning steps can be performed in other processing plants or at the farm itself (e.g. leek is cleaned at the field by removing outer leaves and roots).

Other ingredients will be received in the freezing plant such as technical aids to facilitate the production process (e.g. disinfection products for water treatment), additives (e.g. ascorbic acid/citric acid to avoid enzymatic browning), and also packaging materials for the frozen FVH. Bulk frozen FVH, pieces of frozen meat/fish, sauces, etc. from other facilities can be purchased to process them further to a final product. These other ingredients will be subjected to a reception/inspection and be stored in appropriated conditions (e.g. dry and ambient storage or frozen storage).

After their reception and inspection, fresh FVH can be stored for short time until processing starts. This storage step can be either outside or inside the freezing plant. However, as short intermediate times are aimed for, ambient temperatures will be used in most of the cases. Only in the case of pre-conditioned fresh produce, storage at refrigerated temperatures is needed.

The first steps of processing (=preliminary preparation steps) will highly depend on the type of FVH commodity and may include (Figure 1):

- **Insect removal** in case of leafy greens or beans/peas mainly using rotating drums.

- **Pre-washing** to remove soil or foreign objects such as weeds, seeds, etc.

- **Destoning** to remove stones or other physical hazards from the bulk fresh commodities as potatoes and carrots (by water floating or mechanical action).

- **Pre-cutting including removal of outer leaves and ends** to remove the parts of the fresh FVH which are not used in the final product such as stems of leafy herbs (e.g. basil), end of beans, outer leaves of leek, etc. through mechanical or human intervention.

- **Peeling** (to remove peel) by leaching (using enzymes (e.g. apples)), steaming (e.g. potatoes) or mechanical action (abrasive or carborandum peeling) (e.g. carrots).

- **Size reducing operations**: to make the FVH smaller or appropriate to the size and shape of the final frozen product (e.g. spinach can be left in whole leafs or can be shredded which will end as cubes of frozen shredded spinach). The size of the fresh or frozen FVH subjected to size reduction operations should be as homogeneous as possible. Many different methods and equipment can be identified to perform this production step such as cutting, trimming, shredding, grating, chopping, slicing, chipping. depending on the type of commodity and the desired size/shape. More the mechanical activity and smaller the generated pieces of FVH are, more the release of exudates from cells can be expected and the higher the organic material

of the processing water. In many cases, FVH will be cut after blanching to avoid enzymatic browning or to avoid excessive loss of nutrients.

- **Optical sorting** can be done visually (human interference) but often more sophisticated sorters are applied for colour or shape sorting.

3.2.2. Washing

Washing is conducted in different steps of the process and different types of water quality might be used. The washing step aims to eliminate general field dirt and debris and to remove soil/dust from the product. It also has an impact on the microbiological load of the fresh FVH. Washing of FVH can be achieved by simply spraying the commodities with water, although it generally involves the immersion of the product in chilled water (1 to 10ºC) and washing them one or several times to get the product cleaner. Washing tanks might consist of simple water tanks with paddles or rotating drums to immerse the product under water (to avoid floating of the FVH) or modern aeration 'jacuzzi' washing systems where air is injected to get turbulence. It may also act as a cooling step for the FVH to decrease the product temperature (Gil et al., 2015).

3.2.3. Blanching

Blanching is a thermal treatment that is mainly used to avoid enzymatic browning. It is usually performed prior to food processing such as freezing. Blanching contributes not only to the inactivation of enzymes associated with browning such as polyphenol oxidase (PPO) and peroxidase (POD), but also affects other quality attributes of products (Xiao et al., 2017). If vegetables are not blanched properly, these enzymes continue to be active during frozen storage, causing off-colours, off-flavours, and toughening (De Corcuera et al., 2007; Rickman et al., 2007; Frati et al., 2016).

In the industrial production process, some FVH commodities are always subjected to blanching such as artichoke, celery, corn, broccoli, cauliflower, courgette, Romanesco cauliflower, peppers, beans and peas, pumpkin, carrots, spinach, celery and endive. As such, the frozen blanched products are stable (no further enzymatic activity during long shelf life in the freezer) (Lin and Schyvens, 1995). If possible, all FVH will be blanched before freezing, as it will extend the shelf life of the frozen product. However, some vegetables do not support the blanching process, mainly due to detrimental effects on the quality of the product. This is the case for many fruits, herbs, onion, olives, capers and cucumber among others. The non-blanched FVH are subjected to enzymatic activity during freezing and consequently will have, a shorter shelf life (Frati et al., 2016).

As indicated in Figure 1, when FVH are not subjected to blanching they receive an additional washing step. The reason is that in both cases (blanching/no blanching) the production line is the same and non-blanched FVH will pass by the blanching equipment. The only difference is the temperature of the water, which for blanching will be hot water while for non-blanching will be cold water. When blanching is not applied, and surely for fruits which are sensitive for enzymatic browning such as apples, sometimes antioxidant solutions such as ascorbic/citric acid or syrups are added in the washing water, as additives, to avoid enzymatic browning (Reid, 1996).

Blanching involves heating FVH rapidly to a predetermined temperature which can vary between 65 and 110ºC and maintaining it for a specified time, typically between 1 and less than 10 min depending on the commodity. Common blanching temperatures range from 70 to 100ºC for a few minutes, whereas steam blanching requires applying steam for longer time period (Ceylan et al., 2017). For example, corn is usually subjected to 96ºC for 100 s while parsley is subjected at 85ºC for 80 s. The time/temperature combinations that might be applied during blanching are critical and depend on the time required for inactivation of POD and PPO, which varies with the type of FVH and the size of the pieces to be frozen. The blanched product is either rapidly cooled or passed immediately to a next production step (Xiao et al., 2017). The time/temperature combinations used in the pre-cooling step also varies across freezing plants but they usually reduce the temperature of the product below 10ºC in 1 min, with a maximum of 2 min.

Based on the information compiled by Frati et al. (2016), the blanching step requires frequent controls as well as the search for defects in organoleptic properties and microorganism's concentration (Williams et al., 1986; Rickman et al., 2007). There are several technologies used by the industry for blanching. The most conventional one is thermal blanching, particularly hot water blanching, however,

other technologies such as steam blanching are also used. Microwave and infrared blanching are novel technologies that have not been widely implemented by the industry (Xiao et al., 2017).

As mentioned above, blanching is mostly performed for enzyme inactivation, but it might also be associated with other effects such as decreasing the microbial load or removal of pesticide and toxic residues. Xiao et al. (2017) summarized the different purposes of blanching in FVH as presented in Figure 4. It should be noted that in the freezing plants in the EU, mostly hot water or steam blanching are applied.

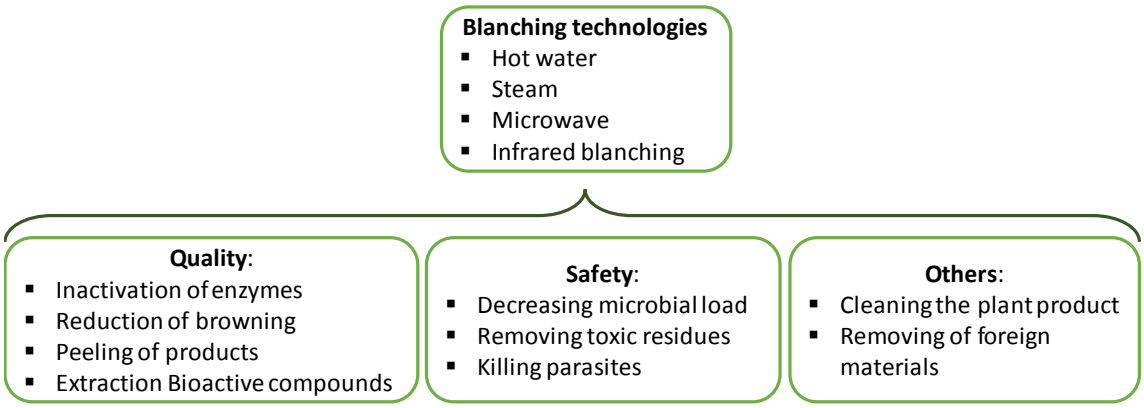


Figure 4: Types of technologies used for blanching and the purpose of blanching (adapted from Xiao et al., 2017)

Blanching can have an impact on the microorganisms present in the FVH by reducing foodborne pathogens associated with FVH such as *L. monocytogenes* (Bozkurt et al., 2015; Ceylan et al., 2017). In a recent study, different vegetables (i.e. peas, spinach, broccoli, potatoes and carrots) were inoculated with approximately 10^8-10^9 CFU/g *L. monocytogenes* and *Salmonella*, respectively and treated with hot water at 85 and 87.8ºC and steam at 58 and 96.7ºC for up to 3.5 min. More than 5 log reductions were observed within 0.5 min of hot water treatment at 85 and 87.8ºC on all vegetables. However, longer treatment times and higher temperatures were required for steam-blanching compared to hot water blanching (Ceylan et al., 2017). Although blanching cannot be considered as a thermal heat treatment to completely inactivate pathogenic vegetative microorganisms such as *L. monocytogenes*, the applied time/temperature combinations during blanching will result in a destruction of a large number of microorganisms in the fresh FVH. Accordingly, it has been reported that blanching can reduce non spore-forming foodborne pathogens by several $\log_{10}$ cycles (Xiao et al., 2017).

3.2.4. Cooling

After blanching, a fast cooling is necessary from the microbiological and sensory (e.g. to remain crispy or keep the colour) point of view. Fast cooling avoids proliferation of microorganisms (i.e. vegetative cells and/or spore-forming bacteria such as *Bacillus cereus* surviving the blanching step). Cold water is usually applied not only to cool the blanched FVH but also to transport the product further in the process by using flumes.

After blanching, the quality of the processing water needs to be carefully controlled to avoid accumulation of organic matter and microorganisms and consequently the potential risk of cross-contamination. Potable water is often combined with a disinfectant agent as a technological aid to retain the water quality. As discussed in Section 3.3.4, the application of a disinfectant is regulated by the national authorities in the EU.

3.2.5. Size reduction operations after blanching or washing

The steps included in a frozen processing line might vary. In some cases, size reduction operations such as cutting/mincing or chopping are performed before washing and/or blanching while in other cases, cutting is done after washing and blanching to avoid browning of the cut surfaces, in particular

for those commodities that are very sensitive to browning such as aubergine. Also, chopping of spinach will be done after blanching the whole leaves and then, the chopped and blanched spinach is shaped into small cubes which will be frozen in the next stage.

Several methods are available for cutting, grating, chopping, shredding, slicing, chipping or mincing fresh FVH into pieces of various shapes and sizes. When a product is chopped or minced very fine, small portions or shapes may be made e.g. cubes of minced spinach or cubes of mashed potatoes. So portioning/forming will be done for finely cut products to get an end product with a desired format e.g. small block of shredded spinach.

3.2.6. Freezing

FVH can be frozen in air blast or cryogenic freezers. More details on the techniques and conditions used can be found in Reid (1996). The product temperature quickly drops to –18°C to stabilise the products and to avoid microbiological activity. To get 'individually quick frozen' products (IQF) a waving conveyor belt moving the FVH into the freezing tunnel is used. Freezing breaks the membrane structure of cells, which cause enzyme dislocation. This can trigger enzymatic reactions independent of frozen storage temperatures, and lead to sensory deteriorations such as discoloration or off-odour formation. In the freezing tunnels, depending on the applied technology (e.g. air blast or cryogenic freezers) and on the specific FVH, a temperature cycle can be introduced between –30 and –40°C followed by a very short cycle at high temperature (e.g. 35–40°C) to avoid excessive ice in the freezing tunnel.

In some cases, FVH are glazed before deep freezing. Glazing is a technique where a protective thin layer of ice is formed on the surface of a frozen FVH by spraying it with or dipping it into potable or clean water at a temperature of about 1–10°C. Due to the temperature difference between the FVH and the water, the water transforms directly into a thin ice layer. Glazing protects the frozen FVH from dehydration and/or oxidation during the prolonged storage in frozen conditions. Glazing is always performed before deep freezing; therefore, glazing occurs in the freezing plants during processing. However, frozen FVH can be subjected to a re-processing after a certain period of storage. In this case, frozen FVH can be also subjected to glazing before their re-processing.

3.2.7. Packaging (or repackaging)

Packaging of frozen FVH is done immediately after freezing. Mechanical packaging protects the frozen product from oxygen and thus prevents oxidation during its prolonged storage.

Frozen commodities can be packaged into different pack sizes, which may range from bulk (high volume packages) to consumer size packages. Bulk packaging is used for further business to business activities, e.g. as in the handling facilities, while big packages are used for business to business activities, e.g. for catering and restaurants. Consumer size packages are used for business to consumer activities.

A wide variety of packaging materials, such as flexible plastic foil or coated carton boxes, are used. Vertical-form-fill and seal as packaging technology is highly applied with simple low density polyethylene plastics covering the product. Sometimes oxygen barriers are included in the packaging material, such as polyamide, to protect the product from oxidation during the prolonged storage at –18°C.

3.2.8. Frozen storage and activities in handling facilities

After freezing, frozen FVH are collected in bulk storage such as big recipients or consumer size packages and they can be send out to the next step in the food chain (e.g. distribution centres). Alternatively, frozen FVH can be temporarily stored in freezing rooms before they are sent to the next step of the supply chain as explained in Section 3.2.7. Another alternative is that the final frozen FVH can go to handling facilities, where it will be further processed including mixing or packaging, as indicated in Figure 3. Typical for handling facilities, the frozen FVH product can be mixed with other frozen semi-finished products, such as pieces of meat or fish or other FVH to make a more convenient type of end product. Also packaging sizes may vary from consumer size packages to bigger volumes for further business to business activities.

Finally, storage, distribution and transportation will be conducted, respecting a freezing temperature of a least –18°C. Typical shelf life periods of FVH can vary from several months to 2 years in frozen conditions (< –18°C).

3.3. Risk factors for *L. monocytogenes* contamination of frozen FVH

L. monocytogenes is widespread in the environment and can be found in soil, water, sewage and decaying vegetation (EFSA BIOHAZ Panel, 2018). It can be readily isolated from humans, domestic animals, fresh agricultural commodities, food packing and processing environments (particularly cool damp areas) (Beuchat, 1996; Hellström, 2011; USFDA, 2017). Several studies have highlighted that *L. monocytogenes* persists in manufacturing equipment and in damp areas of the production environment that may develop into niche harbourage sites (Carpentier and Cerf, 2011; Ceylan et al., 2017).

Practical knowledge of the potential sources and food contamination routes pertinent to *L. monocytogenes* can be useful in the development and application of effective control measures throughout the food chain (Lakicevic and Nastasijevic, 2017) but also for outbreak investigation. There are many potential sources of *L. monocytogenes,* and the raw materials from primary production entering the process are considered of great importance for the presence of the pathogen in the finished product (EFSA BIOHAZ Panel, 2018). The key reservoir for *L. monocytogenes* is soil, and it is frequently found in vegetation, forage, water, sewage and farm environments. *L. monocytogenes* can be also found on equipment used for food processing, preparation, storage, and transportation (Thimothe et al., 2004; FSIS, 2014). Once present in the food processing plant, *L. monocytogenes* inevitably finds suitable niches, particularly in damp spots, in which the microorganism can reside well and also multiply; these critical spots may also include hidden or inaccessible surfaces (MAF, 2011).

Most of the available guidelines indicate that *L. monocytogenes* can be located in protected places of food contact surfaces but also in non-food contact surfaces, which in most of the cases are difficult-to-clean. Special attention should be given to the interior of castings bowls, blade spinner, and other difficult to clean food contact areas such as slicers, knives, cutting boards, conveyor belts (mainly post blanch conveyors/vibratory conveyors), gloves joints, gaskets and dead ends among others as potential sources of *L. monocytogenes* (Lakicevic and Nastasijevic, 2017). Refrigeration systems, including drops coming from it, and the freezing tunnels are wet environments that usually favour survival of *L. monocytogenes*. The freeze/thaw zones, where one side is ambient temperature and cleaned regularly and the other side in a freezer/not regularly cleaned, are considered high risk areas. Freezing tunnels are commonly defrosted every 12–48 h depending on the model but the 'new generation' of freezer tunnels do not allow for a full/complete defrost, so once contaminated, it is almost impossible to eradicate bacteria and product remnants. In general, insulated panels represent a big problem once waterlogged, which is common in freezer tunnels because most are extruded panels and not closed cell insulation. Floors also represent a problem, particularly when made of concrete block, which is porous if not sealed impenetrable.

Drains have been classified as a very important potential niche for *L. monocytogenes* in freezing plants. USDA (2017) indicates that typical places where *Listeria* spp. can harbour in a processing plant include floors, especially cracks and crevices, walls, drains, ceilings, overhead structures and catwalks, wash areas, condensate and standing water, wet insulation in walls and around pipes and cooling units, rubber seals around doors, especially in coolers, metal joints, specially welds and bolts and contents of vacuum cleaners. These indirect contact places can contaminate the product by water spread, moisture, and condensation.

Table 1 provides a summary of the potential sources of contamination of frozen FVH considering the different production steps and activities performed in the freezing plants and handling facilities.

Table 1: Potential sources of contamination with *L. monocytogenes* of frozen FVH considering the different activities performed in the freezing plants and handling facilities (non exhaustive list adapted from EN ISO 18593 and USFDA (2017) and based upon expert discussion)

Production activities	Description of category	Potential source of *L. monocytogenes*
Reception	Fresh FVH	Fruits Vegetables Herbs Pre-conditioned FVH
	Other ingredients than FVH	Meat or meat products Fish or fish products Sauces
	Water sources	Recirculated water Recycled water Clean water
General processing activities	Food contact surfaces	Freeze/thaw zones of the freezing tunnel (freezer tunnel infeed and outfeeds (ambient temperatures)) Conveyor belts (mainly post blanch conveyors/vibratory conveyors) Equipment used for size reduction and peeling (e.g. slicers, blenders, peelers) Mixers Filling and packaging equipment Scale weighers (filler machines with non-wash-down capable electronics) Rubber seals in equipment (non-sanitary design equipment with sandwich joints) Containers to collect frozen FVH Utensils (tables, benches, knives, cutting boards) Product to product cross-contamination
	Non-food contact surfaces	Hollow rollers of conveyors Hardware (touch screens, computer panels) Blower fan assemblies in freezing tunnels Any area where condensation can build up (e.g. condensation drip pans) Any areas of damaged equipment not in contact with food or floor Internal panels of equipment Door handles Floors; focus on floor cracks Pools of water on the floor Walls and floor wall junctures Ceilings Overhead structures Catwalks Rubber seals around doors In-floor weighing equipment Forklifts Trolley/trash bins wheels
	Workers	Uniforms Personnel behaviour Gloves, aprons, footwear
Processing activities using water (such as washing, transportation, blanching, cooling)[(a)]	Food contact surfaces	Equipment used for washing (sprayers, tanks, paddles, rotating drums) Equipment used for blanching Equipment used for cooling (water cooling) Equipment to transport products in water (e.g. flumes)
	Non-food contact surfaces	Pools of water on the floor Pipes (to deliver or remove water) Drains Cracked hoses Devices used to introduce air into (washing) equipment

Production activities	Description of category	Potential source of *L. monocytogenes*
		Water treatment equipment and filters Equipment used to store water Equipment used to cool water Water splashing, moisture and aerosols Any equipment not 'true CIP' treatment (CIP=cleaning in place)
	Water sources	Recirculated water Recycled water Clean water
Cleaning and disinfection of premises [(a)]	Non-food contact surfaces	Vacuum cleaners Floor scrubbers Cleaning tools High pressure devices as tubing systems Water splashing, moisture and aerosols due to high pressure
	Water used for cleaning	Recirculated water Recycled water Clean water
Storage	Non-food contact surfaces	Freezing units Cooling/freezing fans in condensers Cold spots where water condenses Evaporators including evaporator drain Rubber seals around doors

(a): All risk factors listed under general processing activities also apply here.

Sections 3.3.1 to 3.3.6 include risk factors for *L. monocytogenes* associated with the environment, personnel, equipment, fresh FVH and other ingredients than FVH as well as production steps.

3.3.1. Mode of production

The production of frozen FVH is highly dependent on the type of the commodity produced by the freezing plant and the length or intensity of the growth season of the commodities. Therefore, depending on the commodity and the region of production, peaks might occur during spring/summer or autumn/winter. The factories are organised to produce large volumes of bulk products for a certain time period. As such, production is more time-based (several batches of the same product are processed after each other without interrupting the production process) than batch-based (a clear differentiation can be made between batches). When the production of a new commodity is starting, often the equipment and machinery needs to be re-arranged and in some cases re-installed (as other machines might be needed for particular production steps).

Freezing plants are full-time processing plants, where cleaning activities/maintenance are performed when the production of one specific product is finished or after a specific time period. Time intervals between cleaning activities might vary depending on the freezing plant or the type of commodity between few hours and several days. Sometimes, parallel production lines are in place processing either with similar products or other products so that cross contamination cannot be fully excluded. The fact that multiple frozen FVH products are produced using the same equipment and/or production environment may favour cross-contamination from product to product.

Persistent *L. monocytogenes* can still be present in the production environment and/or equipment and contaminate next production batches after a standard cleaning and disinfection procedures followed by a period of non-activity. When cleaning operations are not routinely performed at periodic intervals, the risk of contamination by *L. monocytogenes* may increase due to niche formation or building up biofilms.

Typically the production facilities for frozen FVH are not refrigerated and relatively high temperatures can be reached depending on the season, which might also favour the risk of proliferation of *L. monocytogenes* in the environment.

When the processing environment is dry there is limited opportunity for *L. monocytogenes* to increase in numbers, but the presence of moisture will provide the opportunity for bacteria to become established. The production environment of frozen FVH is a wet environment, thus representing ideal

circumstances for *L. monocytogenes* to proliferate as environmental pathogen. Not all parts of the freezing plants are refrigerated, so in one side of the freezing plant, processing steps are performed under ambient temperature (and cleaned regularly) and at the other side of the freezing plant is a freezer, maintained at very low temperature (and not regularly cleaned). A high temperature gradient will be present in the freezing plants, between ambient temperature and freezing temperatures in the freezing tunnels and storage of frozen products. This temperature gradient may provoke condensation and water drip, which may be a potential factor of contamination and favour the growth of *L. monocytogenes.* The latter will be less relevant for handling facilities where all products are already frozen and treated as frozen products.

3.3.2. Fresh FVH and other ingredients

Ensuring that the incoming fresh FVH and other ingredients are not contaminated with *L. monocytogenes* is important. Occasionally fresh FVH can be contaminated at primary production. In the case of pre-conditioned products, the elimination of the non-edible parts of the product is performed in the field or in other processing plants, which reduces the waste in the freezing plants as well as the potential contamination sources such as soil and outer parts of the crops, which might be contaminated with pathogenic microorganisms, including *L. monocytogenes*. Therefore, these practices are associated with a potential reduction of risks. However, these pre-conditioned activities may also represent an additional risk of contamination with *L. monocytogenes* due to the equipment or processing environment. Thus, the potential prevalence of *L. monocytogenes* in pre-conditioned FVH may be higher compared to fresh unprocessed FVH (Gil et al., 2015).

It should be taken into account that the production steps in place in the freezing plants may not be able to eliminate *L. monocytogenes* in the FVH as no full mitigation strategy is applied in the production process.

3.3.3. Food and non-food contact surfaces in freezing plants and handling facilities

The processing environment, which includes food and non-food contact surfaces, can be a primary source of *L. monocytogenes* (Table 1). Complex operations with a number of different ingredients and multiple processing lines, which might include different production steps, provide more opportunities for contamination. Contamination of food and non-food contact surfaces could conceivably originate from many sources, including fresh FVH, personnel, packaging materials, etc. and may represent both transient and persistent strains of *L. monocytogenes* (Thimothe et al., 2004).

Table 1 summarizes the potential sources of contamination of frozen FVH with *L. monocytogenes* including numerous non-food contact surfaces, which can contaminate the product by water splashing, moisture, condensation and aerosols. Non-food contact sources should be intensively sampled before they can be discarded as a potential source of contamination.

It is important to understand that the prevalence of *L. monocytogenes* in samples from the FPE is highly affected by the sampling site, time of processing and type of food processed (Hellström, 2011). Although the prevalence of *L. monocytogenes* has been reported to decrease after cleaning, the bacterium can be found even after sanitation (disinfection), which indicates its persistence and the insufficient efficacy of the established cleaning and disinfection systems (Norton et al., 2001; Hellström, 2011). *L. monocytogenes* is unlikely to be eliminated from food production and it is likely to be found in any processing facility that handles uncooked material at some point, if monitoring is extensive enough (Tompkin, 2002). Surely for most of the equipment such as big size machines (e.g. blanching equipment), equipment performing size reduction operations (e.g. shredders, slicers), or equipment not cleaned every day/regularly (such as freezing tunnels, storage facilities, tubes for water pumping, etc.), dismantling of its parts may be necessary to ensure adequate cleaning and disinfection.

Avoiding cross-contamination between foods and food contact surfaces is thus critical to minimizing the risk of contamination of FVH with *L. monocytogenes* (Crandall et al., 2012; Lakicevic and Nastasijevic, 2017). Special attention should be paid checking areas harbouring *L. monocytogenes* after the routine sanitation process such as hollow roller on conveyor belts. The factory and equipment design should be also taken into account to evaluate potential presence of

L. monocytogenes. In case of non-sanitary design equipment with sandwich joints, rubber junctions a potential site for *L. monocytogenes* accumulation may occur.

3.3.3.1. Biofilms and persistent cells

The ability of *L. monocytogenes* to persist for long periods in equipment and environments of food industries, particularly under adverse conditions, may be related to biofilm formation (EFSA BIOHAZ Panel, 2018). As a saprophyte, *L. monocytogenes* effectively colonises food contact materials and other niches in the FPE. Once residing in a niche, *L. monocytogenes* is hard to eradicate. *L. monocytogenes* adheres to a variety of surfaces of food contact, including polystyrene, polypropylene, glass, stainless steel, quartz, marble, and granite (Silva et al., 2008). However, *L. monocytogenes* is unable to form thick multilayer biofilms with 10^9-10^{12}CFU/cm^2, as occurs with other bacteria commonly found in biofilms, but it adheres to surfaces with populations reaching 10^4-10^7 CFU/cm^2 (Gram et al., 2007).

Most likely, biofilm formation occurs in sites within the manufacturing environment that are hard to reach and sanitise and that accumulate food residues and water for long time periods (Chmielewski and Frank 2004; Verghese et al., 2011).

In biofilms, *L. monocytogenes* is protected from a variety of environmental factors – such as ultraviolet rays, toxic metals, acids, desiccation, salinity and antimicrobials – and it tolerates better high concentrations of disinfectants and sanitisers hampering the decontamination of surfaces (Carpentier and Cerf, 2011).

EFSA BIOHAZ Panel (2018) concluded that *L. monocytogenes* can be detected in most FPEs over time in varying degrees, and a total absence of *L. monocytogenes* in the FPE cannot be expected. This highlights the need for appropriate sampling programmes and corrective actions to prevent *L. monocytogenes* from being transmitted from in-house sources to the product. One approach envisaged in the USA for *L. monocytogenes* control is the 'seek and destroy' concept (Malley et al., 2015).

L. monocytogenes can persist for months or even years in various environmental niches, including chilled food plants (Lundén, 2004; Møretrø and Langsrud, 2004; Keto-Timonen et al., 2007; Schmitz-Esser et al., 2015). Persistence could be due to high adaptive capacity against physical–chemical factors and due to other genetic determinants increasing survival capacity.

It should be highlighted that most of the research on *L. monocytogenes* biofilm formation and persistence in FPEs has been performed in fish, meat and dairy processing plants. Only few studies have focused on fresh FVH processing plants and further research is needed to address the prevalence and persistence of specific *L. monocytogenes* subtypes within the environments of fresh FVH farms and freezing plants. Evidence from two studies (Sant'Ana et al., 2012; Abeysundra et al., 2017) suggest that *L. monocytogenes* strains isolated from fruits and vegetables were able to form biofilm on food contact surfaces (stainless steel) under laboratory conditions. In one of the two studies, serotypes 1/2b showed a significant adherence capacity when compared to serotypes 4b (Sant'Ana et al., 2012). In general, the majority of strains isolated from frozen vegetables (final product) at the processing plant level seem to belong mainly to serotypes 1/2a followed by 1/2b, 1/2c and 4b (Ballesteros et al., 2011). It was also shown by Duvenage and Korsten (2016) that *L. monocytogenes* reveals capacity to grow on food contact surfaces (vinyl coupons) even under poor nutrient concentrations and at different condition of temperatures.

3.3.3.2. Air, moisture, condensation and aerosols

Moisture, splashing, aerosols, condensation and air can act as a transport vehicle for *L. monocytogenes* to move from biofilm/niche/non-food contact contamination towards the food products. Therefore, air movement and ventilation systems will be important to control.

Due to the use of water and intensive movement of water during washing, blanching and transportation of FVH in production lines, aerosols can be formed which may be redistributing potentially the contamination from non-food contact sources towards food contact surfaces and eventually also towards the food itself. Direct splashing of water from non-food contact surfaces

towards food contact surfaces as may occur during cleaning activities (spraying water under pressure from floors) may also provoke recirculation of the *L. monocytogenes*.

3.3.4. Water sources used in freezing plants and handling facilities

Water is commonly used in the freezing plants as most of the products are washed, blanched, glazed, cooled or transported using water. Water used in direct contact with food products should be potable water, where necessary, as referred by the EU Hygiene Regulation (EC) 852/2004[3], limiting the potential sources of food safety hazards. However, the water quality used in the freezing plants can deteriorate very quickly depending on the production step.

Processing water within the washing tanks or flume systems is characterised by high loads of organic matter including debris, soil, exudates and microorganisms among others (Gil and Allende, 2018). Even though the washing tank is constantly refilled with cold potable water, the accumulation of organic matter and microorganisms in the washing tank cannot be avoided. Processing water is usually recirculated for a certain period of time, which means that the same water can be used to wash large amount of product within the same washing system. The microbiological quality of the recirculated water should be well maintained using an optimal disinfection system to avoid cross-contamination. Cross-contamination occurs during washing when contaminated FVH are washed in processing water, contaminating the water, and thereafter if uncontaminated FVH is washed in the same water there is a high risk of contamination. Therefore, processing water could be the source of microbial contamination or could spread contamination if adequate water quality and/or use of disinfectant agents are not maintained at all times.

Some freezing plants might be able to use recycled water, to save water and energy. The term 'recycled water' refers to the water that is recuperated from the production process and which is usually treated before reuse in any other processing step of the production system. If recycled water is not properly treated to guarantee its microbiological quality, it also represents a potential source of contamination.

As mentioned, water quality is critical to prevent microbial and chemical risks in any processing operations where frozen FVH are in contact with processing water. In most of the cases, the microbial quality of the processing water can be maintained using a disinfection agent. The use of disinfectants depends upon national policies for their approval (EFSA BIOHAZ Panel, 2014). If a disinfectant is not used, washing of FVH relies on continuous addition and refreshing of washing baths with large volumes of potable water, up to 40 L/kg of fresh FVH, to minimise the accumulation of microorganisms in the water and transfer of microorganisms from the water to the products. In some instances, the pre-wash is done with showers to avoid accumulation of organic matter and microorganisms in the processing water. Accumulation of organic matter in the processing water is highly dependent on the type of product being washed. A high concentration of organic matter might influence the efficacy of the applied disinfection agent, as they will be rapidly consumed.

A key element is the design and construction of the drains. Drains should function adequately and should be adequately accessible for cleaning. Trench drains should not be installed in areas where FVH are processed. When possible, it is recommended to replace existing trench drains with enclosed plumbing. In a study carried out by Thimothe et al. (2004) in a smoked-fish factory, *L. monocytogenes* was found in 23.7% of the samples taken from drains, even though floors and drains were cleaned daily and high pressure was not used to clean the drains. They also found that *L. monocytogenes* was infrequently isolated from food contact surfaces, even in plants that had a relatively high number of positive samples in drains and other non-food contact sites. Water used during washing and cleaning, particularly the use of high pressure hoses, will contribute to spreading the bacteria around the processing area (MAF, 2011).

3.3.5. Workers behaviour

Personnel entering in the area where frozen FVH are being processed or exposed to their environment might represent a source of contamination. Special attention should be given to their hands, gloves and uniforms but also to the use of uniforms in low and high-care areas. The use of cleated footwear

[3] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. OJ L 139, 30.4.2004, p. 1–54.

might also represent a risk because this type of shoes can collect particles of dirt or other waste from inside and outside the plant (USFDA, 2017).

Human behaviour plays an important role for instance when workers are crossing from low-care areas to more hygienic areas without any interventions, which might lead to cross-contamination. The use of foamers or footbaths containing sanitisers has been recommended to reduce contamination. A foamer delivers an automatic spray of foam disinfectant on the floor where personnel enter the clean area. A footbath is usually a low flat container, or a water tight recess in the floor, that has a non-slip surface and is filled with a suitable sanitiser (USFDA, 2017).

The personnel using utensils such as spatulas or tongs or wearing gloves should avoid any potential contamination of the frozen FVH. Even though contact between hands and food or packaging material might be necessary under a wide variety of circumstance, special attention should be given to avoid any contamination (USFDA, 2017).

3.3.6. Absence of barriers between fresh and frozen FVH and barriers in production areas

The production process of frozen FVH is a continuous on-line process. Therefore, the principle for forward movement of products could not be a problem. However, as several products and processing lines can run in the same time period and in the same building or production hall, cross-contamination from one line to another line cannot be excluded via water, air, aerosols, workers, moving equipment etc. In the freezing plant, there are low-care areas where the fresh FVH are received, stored prepared for further processing. After washing, blanching, cooling and final preparation steps, more hygienic areas are needed to avoid product contamination (direct or indirect). In fact, the antimicrobial activity of blanching might constitute a risk factor because of cross-contamination of post-blanched products, which show a reduction in their natural microbiota, and might facilitate establishment and proliferation of *L. monocytogenes* mostly because of a reduction in competitive antagonism. Many studies have evidenced that the behaviour of *L. monocytogenes* is defined by its surrounding microorganisms (Zilelidou and Skandamis, 2018). Lianou and Sofos (2007) reported that the absence of food microbiota leads to fast proliferation of *L. monocytogenes* in hot-smoked fish products. Therefore, after blanching, cross-contamination from the production environment becomes more important due to the disruption of the natural ecology of the products.

Based on the importance of the drains in the persistence of *L. monocytogenes*, pipe lines and sinks need to be organised from higher hygiene areas to lower hygiene areas so that no cross-contamination from waste water may occur. Rolling materials, such as trash bins, or equipment could also move from low-care areas towards high-care areas and redistribute the environmental contamination with *L. monocytogenes*. Same with air and ventilation systems which should blow air from high-care to low-care areas to avoid redistribution.

3.4. Monitoring of *L. monocytogenes*

Monitoring and sampling to detect *L. monocytogenes* in a freezing plant or handling facility for frozen FCH can have several objectives such as those described by Uyttendaele et al. (2018):

- batch sampling (product sampling) can be conducted to be able to judge on acceptance or non-acceptance of a batch of fresh FVH and/or finished products;
- surveillance sampling (product sampling) has the objective to seek for prevalence of pathogens (e.g. official monitoring of competent authorities, food products on the market);
- environmental sampling (food contact surfaces) can be organised as verification of routine cleaning and disinfection activities, and
- risk based product and environmental sampling can be conducted in the frame of verification of the prerequisite programmes, HACCP system and broader food safety management system.

The objective of this document is to offer monitoring and sampling in the frame of MST to identify the points of contamination in a processing plant (freezing plant or handling facilities) where *L. monocytogenes* can be harboured and potentially contaminate the food products. MST is a set of techniques which are used to identify the sources (point of origin) of bacteria. MST is also commonly

referred to as faecal source tracking due to the fact that the principle itself was initially developed (and is still largely applied) for identifying the origin of faecal bacteria in contaminated waterways. Application of the principle of MST has been extended over time to other sectors including the food and drink, biotechnology, medical and pharmaceutical industries (Uyttendaele et al., 2018).

As discussed in Table 1, numerous points in the freezing plants and/or handling facilities are identified as potential harbour for *L. monocytogenes*.

To perform an effective MST analysis, a concerted effort should be made to plan the sampling around production batches and environmental CSSs i.e. sampling the same production batch starting from the fresh FVH or ingredients other than FVH used for each batch, through the intermediate products and finally to the final packaged products arising from those batches. These product samples need to be linked to environmental samples taken from food and non-food contact surfaces (see Table 1) and water samples (where necessary, see Table 1). As addressed in Section 3.3.1, it is common that no batch-to-batch production with clear separation between batches of raw materials is occurring in this type of industry due to the continuous type of production. Therefore, a production batch needs to be defined by the industry e.g. 1 production day or 2 production days, where no breakpoint is foreseen to differentiate products (mostly also linked with an intermediate cleaning). From the long list of potential sources of *L. monocytogenes* (in Table 1) a short list of CSSs needs to be elaborated. The relevant CSSs can be defined based upon critical inspection inside a freezing plant or handling facility on e.g. how the production process is organized, which equipment is applied, how the water flows are running, in which condition equipment/floors are or where condensation is built up. It is commonly advised to select 15 to 20 CSSs starting from the beginning of the production process until the end. As an example, a first CSS may be the fresh FVH, a second CSS may be the conveyor belt bringing the fresh FVH towards the next washing step, and a third CSS may be the environment of water tubes feeding the wash baths, etc. The production process should be followed systematically and products and/or environmental CSSs should be identified. An example of a template to define a sampling plan aiming to identify potential sources of *L. monocytogenes* in freezing plants/handling facilities for frozen FVH is given in Table 2.

When there is a combination of product sampling as well as of environment sampling along a production line during production and repeated over several production days, one can determine more accurately at which point in the food production line the target microorganisms, such as *L. monocytogenes*, are introduced in or onto the product as well as their fate along subsequent production steps. It is recommended to collect duplicate samples at each CSS if possible (and affordable).

Several research studies have evaluated the potential contamination with *L. monocytogenes* in the environment of processing facilities of vegetables (Holvoet et al., 2012; Castro-Ibañez et al., 2016). In these studies, processing plants were visited at least three times in separate days and samples were taken at three sampling times during the same working day including the beginning, in the middle and at the end of the working day. At each sampling moment, samples of the fresh FVH and end product, water (pre-wash water, wash water, rinse water and centrifuge effluent water) and food contact surfaces (workers' plastic gloves, conveyor belts, centrifuge and weighing surfaces) were taken for analysis. At least 9 samples of food product (100 g each) or surface were randomly taken from each sampling point. Other studies are conducted in the frame of food service establishments (Lahou et al., 2012), meat processing companies (Jacxsens et al., 2009; Oses et al., 2012), fish processing companies (Noseda et al., 2013; Thi et al., 2014) and an overview of the usefulness of this type of sampling strategy in the meat and dairy sector is discussed by Jacxsens et al. (2011).

For MST, sampling plans are usually distributed over three to four production batches which are sampled over multiple days to gain insight on variability of contamination and potential building up of proliferation on a certain sampling site (including food products as well as environmental samples) (Uyttendaele et al., 2018). When setting up a sampling plan, samples are usually collected three times per day, usually every 3–4 h or depending on the batch throughput in order to detect building up of contamination.

Table 2: Proposal for defining a sampling plan to identify potential sources of *L. monocytogenes* contamination in freezing plants/handling facilities for frozen FVH

Production day	Sampling Time	Critical Sampling Sites (CSSs) need to be identified based upon critical inspection of the freezing plant/handling facility as well as considering the potential sources of contamination of frozen FVH with *L. monocytogenes* listed in Table 1 Combination of product sampling and environmental sampling (e.g. food and non-food contact surfaces, water)			
		CSS1	CSS2	….	CSS15-20
Production 1 (day x)	Time 1				
	Time 2				
	Time 3				
Production 2 (day y)	Time 1				
	Time 2				
	Time 3				
Production 3 (day z)	Time 1				
	Time 2				
	Time 3				

This sampling plan allows to gain the maximum information and to gain insight in the potential variability of the contamination sources. It leads to the collection of a large number of samples. As a possible example for a production line under investigation, 20 CSSs in 3 production days at 3 sampling times during each production day using duplicate sampling result in 360 samples. If there are 3 production lines under investigation, these would represent a total of e.g. 1,080 samples. However in some cases a higher number of CSSs may be needed leading to an increase of the total number of collected samples. It is important to get information on the potential building up of a contamination and also on the potential variability of the contamination.

Two analytical methods (detection and enumeration of *L. monocytogenes)* for food products and the food production environment can be used to identify potential risk factors that allow the detection of potential spots of *L. monocytogenes* cross-contamination towards the food products. There are two European and International Standard methods for detection and enumeration of *L. monocytogenes* in food, i.e. EN ISO 11290-1 and EN ISO 11290-2, respectively. It is important to note that for the identification of *L. monocytogenes* from food products and environmental samples the detection method (with highest sensitivity) should be first applied. In case of a positive result, further characterisation of the *L. monocytogenes* strains should be done and the enumeration method may be used only for fresh or frozen FVH. Strain characterisation based on the subtyping is needed for further matching between isolates from positive samples and/or those found in the human clinical samples. The use of the enumeration method can be interesting to assess the level of contamination of fresh or frozen FVH. The use of *L. monocytogenes* enumeration method in the analysis of environmental samples is not recommended, as swabbing does not detach all bacterial cells and the proportion of detached cells is unknown and variable. In addition, *L. monocytogenes* cells are not evenly distributed on a surface and comparisons of results from large and small areas would thus be invalid (EURL *L. monocytogenes*, 2012).

The EN ISO 11290-2 for enumeration of *L. monocytogenes* in a food product is characterised by a limit of enumeration of 10 CFU/g (1 mL inoculum from the initial suspension used) or 100 CFU/g (0.1 mL inoculum from the initial suspension used). The limit of detection of the EN ISO 11290-1 standard method is 1 CFU/25 g or 1 CFU/sponge or device. In the case of processing water, the limit of enumeration will depend on the volume filtered but 1 CFU/100 mL is the standard. In order to improve the sensitivity of detection of *L. monocytogenes* in the different types of samples (food, processing water and environment), particularly in the scope of support to listeriosis outbreak

investigations, the detection method should be preferred to the enumeration method. Moreover, the detection method is particularly relevant when *L. monocytogenes* is present in small numbers or in stressed conditions or accompanied by considerably larger numbers of other microorganisms, because the detection method includes pre-enrichment and enrichment steps facilitating the growth and resuscitation of *L. monocytogenes*.

For environmental monitoring, as suggested by Codex Alimentarius, 'effective monitoring programmes may also involve testing for *Listeria* spp. as their presence is a good indicator of conditions supporting the potential presence of *L. monocytogenes*' (CAC, 2007). Nevertheless, when the purpose of *L. monocytogenes* monitoring is to support listeriosis outbreak investigations aiming to track the source of *L. monocytogenes* in the FPE, testing for *L monocytogenes* is recommended.

The philosophy of the proposed approach is illustrated in Figure 5, which shows a fit-for-purpose sampling strategy to support outbreak investigation in freezing plants and/or handling facilities for frozen FVH aiming to identify the point of contamination with *L. monocytogenes* in these facilities and to establish links between human clinical isolates, isolates from implicated FVH and from the FPE.

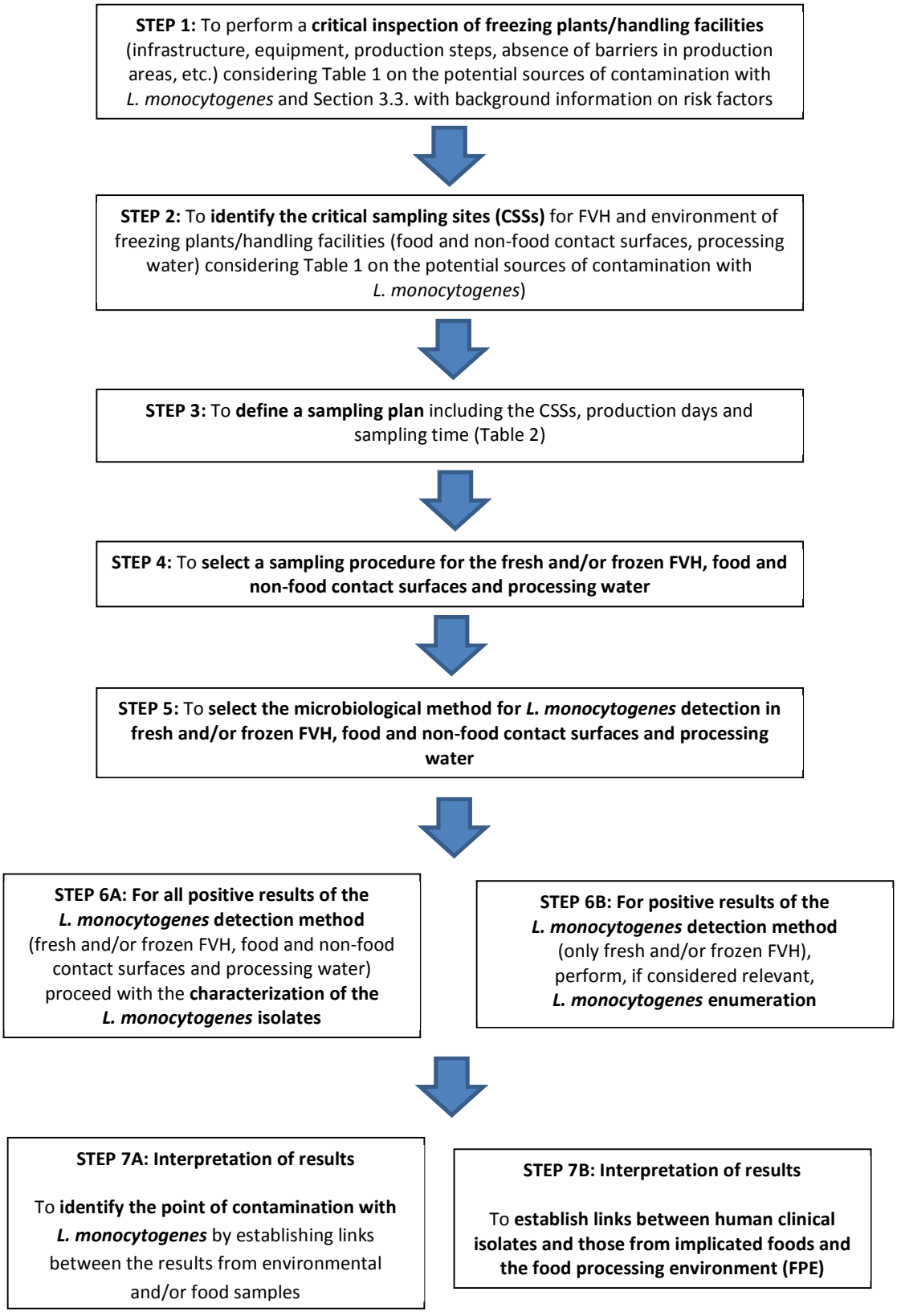


Figure 5: Example of a fit-for-purpose sampling strategy in freezing plants and/or handling facilities of frozen fruits, vegetables and herbs (FVH) aiming to support outbreak investigation

3.4.1. General considerations for bacteriological testing

In general, it is recommended that all bacteriological testing should be conducted in a laboratory that meets the following criteria:

• The laboratory should be physically separated from the food production facility to prevent cross-contamination.

• The laboratory should be staffed by personnel with training and experience in analytical microbiology techniques to ensure that tests are performed correctly and that all appropriate safety precautions, including appropriate waste disposal, are followed.

• Testing should be carried out by laboratories running quality assurance systems or, better, accredited under the EN ISO/IEC 17025.

If microbial analyses are done by the manufacturer, the laboratory facilities, personnel, and quality management system should meet the above mentioned criteria and ensure that testing provides reliable information for food safety management and does not create food safety hazards.

3.4.2. *L. monocytogenes* detection in fresh and frozen FVH

This section focuses on the detection of *L. monocytogenes* in fresh and frozen FVH when sampled inside the freezing plant or inside the respective handling facilities.

3.4.2.1. Food sampling procedure

The collection of samples of fresh and frozen FVH should be done according to the standard CEN ISO/TS 17728:2015 for sampling techniques for microbiological analysis of food samples. The mass of the collected samples should be sufficient to allow microbiological examination and be taken from several units of the sampled food (e.g. 5 units or an equivalent to 100 g).

Sampling techniques shall not modify the natural microbiota of the product (such as via contamination from sampling instruments or the environment). Concerning the sampling of fresh and frozen FVH products, it is necessary to use equipment and a procedure appropriate to the physical presentation of the product (packaged or bulk).

3.4.2.2. Preparation of the initial suspension

General recommendations

The preparation of the test sample should be done in accordance with the specific international standard dealing with the product concerned (see EN ISO 6887-4).

For the fresh FVH, the preparation of the initial suspension should be done in accordance with the Standard EN ISO 6887-4.

Specific recommendation for the preparation of the suspension for the analysis of frozen products

For the analysis of frozen samples, the preparation of the initial suspension should be done in accordance with the Standard EN ISO 6887-1.

For the preparation of the initial suspension, samples shall be tested as quickly as possible after thawing, even if the product is still partially frozen when taking the test portion. Addition of the diluent at ambient laboratory temperature will facilitate full defrosting. Defrosting in a temperature-controlled water bath or under running cold water is not recommended as this can result in contamination of the sample if the packaging is not completely watertight.

Specific recommendation for the preparation of packaged products

Specific recommendations can be proposed for packaged products. Soft packaging is to be removed or opened aseptically using scissors, knives or scalpels, while rigid packaging (cans, glass containers, etc.) is to be opened using appropriate tools under aseptic conditions. All operations, before and after opening packaged foods, shall be carried out aseptically to avoid external contamination. Film-

wrapped portions of food are to be opened on trays carefully by peeling off the packaging film so the food can be exposed for sampling.

Preparation of multi-component products

For multi-component products, test portion sampling should be carried out by taking amounts of each component representative of their proportions in the initial product. Homogenising the whole laboratory sample is also possible as this will provide a more homogeneous test sample for subsequent taking a test portion. It may be necessary to mince or to grind the laboratory sample.

3.4.2.3. Microbiological methods for the detection and enumeration of *L monocytogenes* in fresh FVH and frozen FVH product

Standards methods

According to the EN ISO 11290 part 1 standard the detection of *L. monocytogenes* necessitates four successive stages:

- primary enrichment in a selective liquid enrichment medium with reduced concentration of selective agents (half-Fraser broth);
- secondary enrichment with a selective liquid enrichment medium with full concentration of selective agents (Fraser broth);
- plating out and identification in selective, differential and chromogenic culture media;
- confirmation of presumptive *L. monocytogenes.*

This standard has been validated for test portions of 25 g. A larger test portion may be used if a validation/verification study has shown that this larger test portion size has no adverse effect on the detection of *L. monocytogenes*. Verification for the effect of larger test portion size or pooling test portions can be conducted in accordance with the protocol described in EN ISO 6887-1:2017, Annex D (verification protocol for pooling samples for qualitative tests).

Isolates which are confirmed to be *L. monocytogenes* may be sent for further characterization (typing) to a recognized National Reference Laboratory (NRL) or to the European Reference Laboratory for *L. monocytogenes* (EURL *L. monocytogenes*).

In order to characterise the concentration of *L. monocytogenes* in a product, an enumeration method can be carried out. For the enumeration, the European and International Standard method for enumeration of *L. monocytogenes* in food EN ISO 11290-2 is recommended.

According to the EN ISO 11290-2 standard the enumeration of *L. monocytogenes* necessitates five successive stages:

- preparation of the initial suspension in an appropriate diluent;
- surface plating on Agar *Listeria* according to Ottaviani and Agosti (ALOA);
- incubation of the Petri dishes;
- confirmation of presumptive colonies;
- calculation of the number of *L. monocytogenes* per gram from the number of confirmed colonies.

3.4.3. Monitoring for *L. monocytogenes* in the environment of freezing plants and handling facilities for frozen FVH (including processing water)

According to the requirements of EC Regulation 2073/2005[4], samples shall be taken from processing areas and equipment used in food production, when such sampling is necessary for ensuring that the

[4] Regulation (EC) 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. OJ L 338, 22.12.2005, p. 1–26.

microbiological criteria are met. For environmental sampling, the Standard EN ISO 18593 shall be used as the reference sampling techniques from surfaces of food environments. *L. monocytogenes* detection analyses of environmental samples should be performed according to the EN ISO standard method 11290-1. In addition, the EURL *L. monocytogenes* has drafted a document providing guidance on sampling processing areas, which is specific to *L. monocytogenes* (EURL *L. monocytogenes*, 2012).

FBOs manufacturing fresh or frozen FVH, which may pose a *L. monocytogenes* risk for public health, shall sample the processing areas and equipment for *L. monocytogenes* as part of their sampling scheme. As discussed in Section 3.4, the objective of the monitoring described here is to perform MST and to identify potential spots harbouring *L. monocytogenes* in freezing plants or handling plants.

3.4.3.1. Sampling procedure for food and non-food contact surfaces (EN ISO 18593)

Sampling sites and areas, time of sampling and sampling techniques should be selected according to risk-based principles and should relate to the higher probability of detecting contaminated surfaces during food processing, when measuring the hygiene of specific production steps or the entire process as appropriate.

Sampling site

L. monocytogenes can be found on visually clean surfaces but are most frequently found on wet and soiled places where the bacteria are able to grow and persist (Carpentier and Cerf, 2011).

L. monocytogenes finds niches present in the FPE, in which it can persist; these critical spots may also include hidden or inaccessible equipment surfaces (MAF, 2011). Hard to reach places such as holes or crevices in fibrous, porous, difficult-to-clean equipment, rusting and hollow materials, are potential harbourage sites that should be sampled. It can be difficult to sample unreachable areas where food debris can collect. These areas should be sampled after dismantling the equipment with the technical team of a FBO.

The choice of the sampling site shall be defined according to historical data linked to each site and after step-by-step examination of the process.

Table 1 in Section 3.3 includes a summary of the potential sources for *L. monocytogenes* contamination in freezing plants and handling facilities for frozen FVH. The food and non-food contact surfaces identified in Table 1 are sampling sites where *L. monocytogenes* harbours and/or may allow a potential cross-contamination towards the food products.

Sampling area

For the detection of *L. monocytogenes*, when the area is accessible, the total sampled area should be as large as possible to increase the probability of detecting this microorganism. In this regard, it is recommended to sample between 1,000 cm² and 3,000 cm² (i.e. 0.1 m^2 to 0.3 m^2) when the surface is large and accessible. For the sampling of hard-to-reach or small areas, stick swab should be used to sample approximately 100 cm^2, when possible. However, it should be taken into account that many sampling sites may not be square areas but some sites may represent narrow cracks that could be sampled over multiple meters.

Time of sampling and frequency

As this technical report focuses on the identification of the point of origin of a microbial contamination, the described sampling procedures should be performed as exhaustively as possible to cover the largest number of CSSs and samples per CSS. Whenever feasible, samples should be taken at different sampling times including several hours after the start of the production of frozen FVH, when accumulation of contamination is more likely to occur. In an outbreak investigation trace-back situation, collection of more than 500 samples may not be unusual. A considerable number of samples may also be taken in non-processing areas (e.g., receiving areas, storage areas, particularly if areas are wet).

The detection of *L. monocytogenes* can be difficult when environmental samples are taken immediately or soon after cleaning and disinfection. Cells can still be alive but non-culturable, as a result of injury caused by the chemical agents used for cleaning and disinfection, and may not be

easily detectable. Furthermore, cells remaining in harbourage sites despite cleaning and disinfection can also be undetected, while they are more accessible to sampling once dislodged during processing because equipment vibrates and/or because foods and liquids come in contact with harbourage sites (Tompkin, 2004).

Therefore, to increase the probability of detecting a persistent strain, sampling should be performed during processing, at least 2 hours after production or at the end of production runs, i.e. before cleaning and disinfection. In processing lines where food products are manufactured from fresh products which are not subjected to a treatment that reduces the level of microorganisms, *L. monocytogenes* in a surface sample taken during the processing run may originate from these fresh products as well as from the places where *L. monocytogenes* cells can persist in the FPE (EURL *L. monocytogenes*, 2012).

Sampling techniques

Various sampling techniques are described in EN ISO 18593.

For the sampling of hard-to-reach, small areas (≤ 100 cm^2) stick swab should be used to sample.

For the sampling of large surfaces (> 100 cm^2), sterile cloths or sponges should be used.

After sampling, the surface is cleaned and/or disinfected, if necessary, to avoid traces of nutrients, moisture, chemical or physical elements resulting from the sampling procedure remaining on the sampled surface. This can be done with sterile wipes, moistened with alcohol.

Diluents

In case of easy-to-reach areas which are sampled during or at the end of processing, simple diluents containing no neutraliser should be used to moisten stick swabs and the other wipe sampling equipment. Recommended diluents are: peptone solution at 1 g/L, peptone saline or quarter-strength Ringer's solution. Phosphate buffered diluents are not recommended to be used for cells stressed by hostile conditions of food processing premises (salt, acid, cleaning and disinfecting products etc.) since they may have a deleterious impact on their culturability.

When no residual disinfectant is expected on the area, it is recommended not to use a neutralising diluent. A neutraliser used to quench residual disinfectant can have a slight deleterious impact on bacterial cells and it is likely that such an impact would be greater when cells are stressed. Fraser broth or half-Fraser broth should not be used in place of a diluent since they could favour growth of *L. monocytogenes* in the processing site.

In any areas where residues of disinfectants are expected, or when samples are taken immediately after disinfection, neutralising diluents should be used to moisten stick swabs or other wipe sampling equipment. An appropriate neutraliser for all situations (a 'universal neutraliser') cannot be prescribed. A number of neutralisers are recommended in EN 1276, EN 1650, EN 13697 and EN 13704.

Sampling devices

The use of the different sampling devices is described in EN ISO 18593.

- Stick swab method

Stick swabs should be used to sample hard-to-reach small areas (e.g. inside hollow rollers, motor housing, knives). It is recommended to use sterile disposable templates to prevent the transfer of contamination and/or disinfectant compounds. The size of the sampled area shall be approximately known and/or the sampling site well described.

To use a swab, remove the stick swab from the sterile wrapping and, if needed, moisten the tip by immersing it in a tube containing the diluent/neutraliser and press the tip of the swab against the wall of the tube to remove excess diluent/neutraliser. Place the tip of the swab on the surface to be examined and streak an estimated area of e.g. ≤ 100 cm^2, while rotating the stick swab between thumb and forefinger. For flat surfaces, the sampling should be performed horizontally and vertically. For hard-to-reach small surfaces, make sure to sample the entire described site including crevices, gaps, surface connections, etc. Return the stick swab in the tube with the diluent/neutraliser. Make sure the tube is closed so that the swab stays moist until the analysis.

- Sponge/cloth method

A sponge or cloth should be used to sample large areas (floors, walls, ceilings, conveyor belts). In contrast with stick swabs they can be rubbed more vigorously over surfaces and are highly absorptive. The cloth or sponge should have been moistened with a sufficient quantity of diluent/neutraliser. In the event that the area to sample is wet, a dry sponge/cloth may be used, unless neutralisers are needed. In the event that the area to sample is dry, a moistened sponge/cloth must be used, except if moisture cannot be introduced in the processing area. In order to increase the recovery, it is recommended to use moistened sponge/cloth.

To use a sponge or cloth, open the plastic bag or container containing the cloth or sponge. Remove aseptically the cloth or sponge for example with sterile forceps and/or a sterile gloved hand, or grab the sponge/cloth through the bag and pull the reversed bag over the hand. Sample the chosen surface horizontally and vertically using even and firm pressure, changing the face of the cloth or sponge and ensuring the whole area is sampled. Return the cloth or sponge to the plastic bag or container. Close the plastic bag or container in a manner that will ensure no cross-contamination.

3.4.3.2. Sampling procedure for the processing water

According to Regulation (EC) 2073/2005, no method is indicated for the analyses of water used in food processing. Additionally, no specific EN ISO methods exist for the detection of *L. monocytogenes* in processing water.

Several research studies have evaluated the potential contamination with *L. monocytogenes* in the environment of processing facilities, including processing water. In these studies, samples of processing water, including pre-wash water, wash water, rinse water and centrifuge effluent water were taken for analysis (Holvoet et al., 2012; Castro-Ibañez et al., 2016). Water samples were collected into sterile 1-litre Schott bottles according ISO 19458:2006. For the analysis of *Listeria* spp. and *L. monocytogenes* in irrigation and processing water, filtration and direct plating have been proposed (López-Gálvez et al., 2014; Zhou et al., 2015).

3.4.3.3. Storage and transport of samples

Guidance on storage and transport of samples is given in EN ISO 18593.

In the case of food and non-food contact surfaces (stick swab/cloth/sponge method), the delay between sampling and testing should be as short as possible. The samples should preferably be cooled before being put into insulated transport containers, and transported at 1–8°C. Samples should preferably be examined within 24 h from sampling. If the testing is delayed after receipt in the laboratory, the samples shall be stored at 3°C ± 2°C for a maximum of 48 h from sampling.

For processing water, samples of about 1 L each should be taken in sterile containers and neutralised to stop the action of any disinfectant if present. Then, water containers should be transported as soon as possible under refrigerated conditions to the laboratory located in the processing plant. In most of the cases, 1 L processing water is mixed in the containers with enough (0.5–1.5 g) solid sodium thiosulfate pentahydrate (Scharlau, Barcelona, Spain) for the quenching of disinfectant residuals (López-Gálvez et al., 2018).

3.4.3.4. Microbiological methods for the analysis of environmental samples (including processing water)

General guidance on microbiological analysis is given in EN ISO 18593 and in EN ISO 11290-1.

For food and non-food contact surfaces, enough diluent/enrichment broth is to be added to cover the sampling device. The exact volume shall be known. Examples of volumes to be used for dilution are 9 to 10 mL for swabs, 90 to 100 mL for sponges and 225 mL for cloths. Thoroughly homogenise the contents by hand or mechanical massaging the sponge/cloth or vortexing the stick swab.

For processing water, in most cases, the filtration method is selected. In the filtration method, volumes between 10 and 100 mL were filtered through 0.45 µm membrane filters using a filter holder manifold (Millipore, Madrid, Spain). ALOA and PALCAM *Listeria* selective agar media have been suggested as culture media for filters and direct plating, and incubated at 37°C for 48–72 h before interpretation of results (López-Gálvez et al., 2014; Zhou et al., 2015).

As mentioned under Section 3.4, the use of *L. monocytogenes* enumeration method in the analysis of environmental samples is not recommended, as swabbing does not detach all bacterial cells and the proportion of detached cells is unknown and variable. In addition *L. monocytogenes* cells are not evenly distributed on a surface and comparisons of results from large and small areas would thus be invalid (EURL *L. monocytogenes*, 2012).

3.4.4. Alternative methods for *L. monocytogenes* detection

Regulation (EC) 2073/2005 (Article 5) indicates the possibility of using alternative methods to those indicated as reference methods, provided they are validated against the reference method as set out in Annex I to Regulation 2073/2005 according to EN ISO 16140-2 as well as certified, for proprietary (commercial) methods. Several alternative methods have been validated according to this EN ISO 16140 protocol for the detection of *L. monocytogenes* in food and animal feed samples ('horizontal methods') and certified by e.g. MicroVal or Afnor Certification bodies.

An overview of these validated alternative methods is available at the relevant websites of MicroVal and Afnor Certification. According to the EN ISO16140-2, the selection of categories and types of matrix used within the validation will depend on the type or group of microorganism and the scope of the validation. If the method is to be applied to a broad range of foods, then at least five categories of food shall be studied. In addition to food, feed samples, environmental samples can be included as additional categories.

Appendices A and B present an overview of the alternative methods that can be performed for the detection of *L. monocytogenes* in fresh or frozen FVH and environmental samples from freezing plants, certified by MicroVal and Afnor validation (according to the data available on their website). These tables only focus on the methods that allow isolation of *L. monocytogenes* strains for further characterisation, e.g. comparison with other strains via WGS in the scope of foodborne outbreak investigations. The description of these alternative methods constantly evolves, so it is necessary to refer to the websites of MicroVal and Afnor Certification.

3.4.5. Characterisation of *L. monocytogenes* isolates

It is necessary to characterise the *L. monocytogenes* isolates from fresh and/or frozen FVH, food and non-food contact surfaces and processing water in order to identify the point(s) of contamination with *L. monocytogenes* in the freezing plant/handling facility and to establish links between human clinical isolates, isolates from implicated FVH and from the FPE.

Subtyping of foodborne pathogen isolates can be used by FBOs as part of their routine environmental monitoring programmes (with every pathogen isolate characterised by subtyping) or can be used as a specialized tool when necessary (e.g., to identify the specific source responsible for the final product contamination). Importantly, molecular subtyping studies on *L. monocytogenes* have also helped identify actual sites ('niches') where *L. monocytogenes* can survive over time in food-associated environments (Simmons and Wiedmann, 2017).

In that way, subtype characterization of pathogen isolates from environmental monitoring in the premises of freezing plants for FVH, processing water, as well as fresh or frozen FVH can provide relevant information for MST. This can be done using well-established molecular techniques such as Pulsed-Field Gel Electrophoresis (PFGE), ribotyping, Amplified Fragment Length Polymorphisms (AFLP), Multi-locus Sequence Typing (MLST), PCR methods or other relevant methods including analysis of Whole Genome Sequencing (WGS).

WGS techniques, when combined with epidemiological information, have shown the potential to attribute relatedness among *L. monocytogenes* strains and thus to establish stronger links between human listeriosis cases and causative foods (EFSA BIOHAZ Panel, 2018). Currently, in the scope of multi-state outbreak investigations in the EU, both PFGE and WGS are used for identification of the point of contamination with *L. monocytogenes* in the food industries and to establish links between human clinical isolates, isolates from implicated foods and the FPE.

4. Conclusions and recommendations

ToR 1. Provide recommendations on the sampling strategies and established microbiological methods most appropriate for maximising the sensitivity of detection of L. monocytogenes *in processing water and the environment of premises producing frozen vegetables, as well as on the final food produced*

ToR 2. Provide recommendations on the identification of critical sampling sites for environmental monitoring of L. monocytogenes *in the processing plants for frozen vegetables. This should take into account aspects related to the persistence and growth niches of* L. monocytogenes

- The recommended sampling strategies and microbiological methods for *L. monocytogenes* detection have the aim to maximize the sensitivity of detection of this foodborne pathogen in the environment of the freezing plants and handling facilities for frozen fruits, vegetables or herbs (FVH). The objective is to identify sources and routes of contamination of *L. monocytogenes* in these production sites. **Seven steps** can be defined for a fit-for-purpose sampling strategy.
- **Step 1:** To perform a critical inspection of freezing plants/handling facilities for frozen FVH (e.g. infrastructure, equipment, production steps, absence of barriers in production areas) considering Table 1 on the potential sources of contamination with *L. monocytogenes* and Section 3.3:
    - Complex operations with a number of different ingredients and multiple processing lines which might include different production steps provide more opportunities for contamination.
    - In the freezing plant, there are low-care areas where the fresh FVH are received, stored and receive their preliminary preparation steps. After washing and blanching, cooling and final preparation steps, more hygienic areas are needed to avoid product contamination (direct or indirect).
    - The relevant critical sampling sites (CSSs) can be defined based upon critical inspection inside a freezing plant or handling facility on e.g. how the production process is organized, which equipment is applied and how the water flows are running.
- **Step 2:** To identify the CSSs for FVH and environment of freezing plants/handling facilities (food contact and non-food contact surfaces, and processing water) considering Table 1:
    - From the long list of potential sources of *L. monocytogenes*, a short list of CSSs needs to be elaborated.
    - A concerted effort should be made to plan the sampling around production batches and environmental CSSs, i.e. sampling the same production batch starting from the fresh FVH or ingredients other than FVH used for each batch, through the intermediate products and finally to the final packaged products arising from those batches.
    - Typical non-food contact surfaces where *L. monocytogenes* can harbour in a freezing plant include: floors, especially cracks and crevices, walls, drains, ceilings, overhead structures and catwalks, wash areas, condensate and standing water, wet insulation in walls and around pipes and cooling units, rubber seals around doors, especially in coolers, metal joints, specially welds and bolts and contents of vacuum cleaners. These indirect contact places can re-contaminate the product by water spread, moisture, and condensation.
    - Regarding food contact surfaces, *L. monocytogenes* is commonly found on equipment used for food processing, preparation, storage, and transportation such as freezing tunnels, castings bowls, blade spinner, slicers, knives, cutting boards, conveyor belts (mainly post blanch conveyors/vibratory conveyors), gloves joints, gaskets and dead ends among others.
    - Once present in the freezing plant, *L. monocytogenes* inevitably finds suitable niches, in particular damp spots, in which the microorganism can reside well and also

multiply; these critical spots may also include hidden or inaccessible equipment surfaces not regularly cleaned or maintained.

- Furthermore, cells remaining in harbourage sites despite cleaning and disinfection can also be undetected, while they are more accessible to sampling once dislodged during processing because equipment vibrates and/or because foods and liquids come in contact with harbourage sites.
- Water is commonly used in the freezing plants as most of the products are washed, blanched, glazed, cooled or transported using water. Processing water can be a potential source for cross-contamination between different batches. Cross-contamination occurs during washing when contaminated FVH are washed in processing water, contaminating the water, and thereafter if uncontaminated FVH are washed in the same water there is a high risk of contamination.
- Water used during washing and cleaning, particularly the use of high pressure hoses, will contribute to spreading the bacteria around the processing area.
- Human behaviour plays an important role for instance when workers are crossing from low-care areas to more hygienic areas without any interventions, which might lead to cross-contamination.

- **Step 3:** To define a sampling plan including the CSSs, production days and sampling time (Table 2):
  - The sampling principle of microbial source tracking (MST) is recommended to identify the point of origin of *L. monocytogenes* contamination in freezing plants/handling facilities for frozen FVH. The sampling procedures described in this report should be performed as exhaustively as possible to cover the largest number of CSSs and samples per CSS to gain insight in the potential variability of the contamination sources.
  - Whenever feasible, samples should be taken at different sampling times, including several hours after the start of the production of frozen FVH, when accumulation of contamination is more likely to occur.
  - As a possible example for a production line under investigation, 20 CSSs in 3 production days at 3 sampling times during each production day using duplicate sampling result in 360 samples which would allow to gain insight in the potential variability of the contamination sources. However in some cases a higher number of CSSs may be needed leading to an increase of the total number of collected samples. In an outbreak investigation trace-back situation, collection of more than 500 samples may not be unusual.
- **Step 4:** To select a sampling procedure for the fresh and/or frozen FVH, food and non-food contact surfaces and processing water:
  - The mass of the collected food samples should be sufficient to allow microbiological examination and be taken from several units of the sampled food (e.g. 5 units or an equivalent to 100 g).
  - To increase the probability of detection of *L. monocytogenes* during environmental monitoring, an area as large as possible should be sampled using sponges or cloths. Conversely, stick swab should be used to sample hard-to-reach small areas. Equipment dismantling to reach the most inaccessible parts of the equipment might be necessary.
- **Step 5:** To select the microbiological method for *L. monocytogenes* detection in fresh and/or frozen FVH, food and non-food contact surfaces and processing water:
  - To improve the sensitivity of detection of *L. monocytogenes* in food samples, particularly in the scope of support to listeriosis outbreak investigations or environmental testing, the EN ISO 11290-1 detection method should be preferred to

the enumeration method. The limit of detection of this method is 1 CFU/25 g or 1 CFU/sponge or device or 1 CFU/100 mL and it allows to isolate *L monocytogenes* when present in small numbers or in stressed conditions or accompanied by considerably larger numbers of other microorganisms.

- The detection of *L. monocytogenes* can be difficult when environmental samples are taken immediately or soon after cleaning and disinfection. Cells can still be alive but non-culturable, as a result of injury caused by the chemical agents used for cleaning and disinfection, and may not be easily detectable.
- Effective environmental monitoring programmes may also involve testing for *Listeria* spp. as their presence is a good indicator of conditions supporting the potential presence of *L. monocytogenes*. When the purpose of monitoring is to support listeriosis outbreak investigations aiming to track the source of *L. monocytogenes* in the FPE, testing for *L monocytogenes* is recommended.
- Several alternative analytical methods have been validated according to the EN ISO 16140 protocol for the detection of *L. monocytogenes* in fresh or frozen FVH and in the environment of freezing plants and handling facilities for frozen FVH.

- **Step 6:** For all positive results of the *L. monocytogenes* detection method (fresh and/or frozen FVH, food and non-food contact surfaces and processing water) to proceed with the characterization of the *L. monocytogenes* isolates (step 6A). For positive results of the *L. monocytogenes* detection method (only fresh and/or frozen FVH), to perform, if considered relevant, *L. monocytogenes* enumeration (step 6B).
  - Subtype characterization of *L. monocytogenes* isolates needs to be done to identify the point of contamination with *L. monocytogenes* in the freezing plants/handling facilities for frozen FVH and to establish links between human clinical isolates, isolates from implicated FVH and from the FPE. This can be done using well established molecular techniques.
- **Step 7:** To interpret the results aiming to:
  - identify the point of contamination with *L. monocytogenes* in the food industries by establishing links between the results from environmental and/or food samples (step 7A);
  - establish links between human clinical isolates and those from implicated FVH and the FPE (step 7B).

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Abbreviations

AFLP	Amplified Fragment Length Polymorphisms
AFNOR	Association Française de Normalisation
ALOA	Agar *Listeria* according to Ottaviani and Agosti
CFU	Colony Forming Unit(s)
CSSs	critical sampling sites
EC	European Commission
ECDC	European Centre for Diseases Prevention and Control
EU	European Union
EURL	European Union Reference Laboratory
EEA	European Economic Area
FBOs	food business operator(s)
FPE	food processing environment
FVH	Fruits, vegetables or herbs
HACCP	Hazard Analysis and Critical Control Points
IQF	Individually Quick Frozen
ISO	International Organization for Standardization
MLST	Multi-locus Sequence Typing
MS	Member State
MST	Microbial Source Tracking
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
POD	Polyphenol Oxidase
PPO	Polyphenol Peroxidase
QMS/FSMS	Quality and Food Safety Management System
ST	sequence type
ToR	Terms of Reference
WGS	Whole Genome Sequencing

Appendix A – Overview of alternative methods for the detection of *L. monocytogenes* in the environment of freezing plants and handling facilities for frozen FVH certified by Afnor validation

Method	Name of the method (Holder)	Validation reference document	Reference method	Certificate number	Scope of validation	Exclusion	Validation food category
Culture media	AL/Agar Detection (detection of *L. monocytogenes*) (Biorad)	EN ISO 16140-2 (2016)	EN ISO 11290-1 (2017)	BRD 07/16 - 01/09	All human food products and production environmental samples		vegetables
Culture media	ALOA ONE DAY (detection of *L. monocytogenes* and *Listeria* spp.) (bioMérieux)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	AES 10/03 - 09/00	All human food products and production environmental samples		fruit and vegetables
Culture media	COMPASS *Listeria* Agar (detection of *L. monocytogenes* and *Listeria* spp.) (SOLABIA SAS)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	BKR 23/02 - 11/02	All human food products and production environmental samples		vegetables and miscellaneous
Culture media	ChromID Lmono Agar (detection of *L. monocytogenes*) (bioMérieux)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	BIO 12/31 - 05/11	All human food products and production environmental samples		vegetable products
Culture media	LESS Plus for *Listeria monocytogenes* + ANSR for *Listeria monocytogenes* or culture media (NEOGEN Europe Ltd)	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	NEO 35/06 - 07/16	All human food products and production environmental samples		vegetables
Culture media	*Listeria* Precis (Detection of *L. monocytogenes* and *Listeria* spp.) (OXOID Ltd, Part of Thermo Fisher Scientific)	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	UNI 03/04 - 04/05	All human food products and production environmental samples		vegetables
Culture media	RAPID'L.mono (Detection of *L. monocytogenes* and *Listeria* spp.) (Biorad)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	BRD 07/04 - 09/98	All human food products and production environmental samples		vegetables
molecular methods	Accuprobe *Listeria monocytogenes* (detection) (bioMérieux)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	BIO 12/04 - 02/95	All human food products and production environmental samples		vegetables and miscellaneous
molecular methods	3M Molecular Detection Assay 5 *Listeria monocytogenes* (Detection of *L. monocytogenes*) (3M)	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	3M 01/15 - 09/16	All human food products and production environmental samples	Samples from primary production	vegetables
molecular methods	ANSR for *Listeria monocytogenes* (Detection of *L. monocytogenes*) (NEOGEN Europe Ltd)	ISO/FDIS 16140-2 (2015)	EN ISO 11290-1 /A1 (2005)	NEO 35/04 - 03/16	All human food products and production environmental samples		vegetables
molecular methods	BACGene *Listeria* Multiplex/*Listeria monocytogenes* (Detection of *Listeria* spp and *L. monocytogenes*) (Eurofins GeneScan GmBH)	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	EGS 38/05 - 03/17	All human food products and production environmental samples		vegetables
molecular methods	BACGene *Listeria monocytogenes* (Detection of *L. monocytogenes* (Eurofins GeneScan GmBH))	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	EGS 38/03 - 01/17	All human food products and production environmental samples		vegetables
molecular methods	BAX System PCR Assay for *Listeria monocytogenes* 24E	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	QUA 18/05 - 07/08	All human food products and production		vegetables

Method	Name of the method (Holder)	Validation reference document	Reference method	Certificate number	Scope of validation	Exclusion	Validation food category
	(Detection of *L. monocytogenes*) (DuPont Qualicon)				environmental samples		
molecular methods	Gene-Up *Listeria monocytogenes* (Detection of *L. monocytogenes*) (bioMérieux)	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	BIO 12/40 - 11/16	All human food products and production environmental samples		vegetable products
molecular methods	GeneDisc *Listeria monocytogenes* (Detection of *L. monocytogenes*) (Pall GeneDisc Technologies)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	GEN 25/08 - 07/10	All human food products and production environmental samples		vegetables
molecular methods	iQ -Check *Listeria monocytogenes* II (Detection of *L. monocytogenes*) (Bio-Rad)	EN ISO 16140-2 (2016)	EN ISO 11290-2 (2017)	BRD 07/10 - 04/05	All human food products and production environmental samples		vegetable products
molecular methods	LUMIPROBE 24 *Listeria monocytogenes* (Detection of *L. monocytogenes*) (EUROPROBE)	EN ISO 16140-2 (2016)	EN ISO 11290-2 (2017)	EUR 15/03 - 12/05	All human food products and production environmental samples		vegetable products
molecular methods	MicroSEQ *Listeria monocytogenes* (Detection of *L. monocytogenes*) (Life Technologies)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	ABI 29/05 - 12/11	All human food products and production environmental samples		vegetables
molecular methods	ThermoScientific SureTect *Listeria monocytogenes* PCR Assay (Detection of *L. monocytogenes*) (OXOID Ltd, Part of Thermo Fisher Scientific)	EN ISO 16140-2 (2016)	EN ISO 11290-1 (2017)	UNI 03/08 - 11/13	Meat products, milk & milk products, seafood & fishery products, vegetables, production environmental samples		vegetables
Immunological tests	TRANSIA Plate *Listeria monocytogenes* (Detection of *L. monocytogenes*) (BioControl)	EN ISO 16140-2 (2003)	EN ISO 11290-1 /A1 (2005)	TRA 02/11 - 03/08	All human food products and production environmental samples		vegetables
Immunological tests	VIDAS *Listeria* Duo (LDUO) (Detection of *Listeria* spp. and *L. monocytogenes*) (bioMérieux)	EN ISO 16140-2 (2016)	EN ISO 11290-1 (2017)	BIO 12/18 - 03/06	All human food products and production environmental samples		vegetables
Immunological tests	VIDAS *Listeria monocytogenes* II (LMO 02)-enrichment 30°C (Detection of *L. monocytogenes*) (bioMérieux)	EN ISO 16140-2 (2016)	EN ISO 11290-1 (2017)	BIO 12/09 - 07/02	All human food products and production environmental samples	raw products	vegetables
Immunological tests	VIDAS *Listeria monocytogenes* II (LMO 02)-enrichment 37°C (Detection of *L. monocytogenes*) (bioMérieux)	EN ISO 16140-2 (2016)	EN ISO 11290-1 /A1 (2005)	BIO 12/11 - 03/04	All human food products and production environmental samples		vegetables
Immunological tests	VIDAS *Listeria monocytogenes* Xpress (LMX)) (Detection of *L. monocytogenes*) (bioMérieux)	EN ISO 16140-2 (2016)	EN ISO 11290-1 (2017)	BIO 12/17 - 02/10	All human food products and production environmental samples		vegetables

Appendix B – Overview of alternative methods for the detection of *L. monocytogenes* in the environment of freezing plants and handling facilities for frozen FVH certified by Microval

Method	Name of the method (Holder)	Validation reference document	Reference method	Certificate number	Scope of validation	Validation food category
Culture medium	ChromID Lmono Agar (LMO-F) (enumeration of *L. monocytogenes*) (bioMérieux)	EN ISO 16140 (2003)	EN ISO 11290-1/A1 (2004)	2010LR35	All human food products and production environmental samples	Fruits and vegetables