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To: Produce WG
Ccfi: Technical Committee, Executive Committee

PRODUCE DECONTAMINATION ASSESSMENT PROTOCOL: PART 1 – ATTACHMENT

CFA has been working for some months with IFR on two papers regarding produce decontamination, intended for publication in a peer-reviewed journal, setting out

1. how to attach bacteria to plant surfaces
2. how to validate decontamination effects of washing

The overall aim is to introduce consistency into experimental approaches.

The first paper is enclosed, which sets out a protocol for researchers to ensure that inoculated organisms are attached prior to attempted detachment, hence avoiding overstating apparent detachment.

The approach was validated by IFR as part of the CFA/Defra-funded AFM234 pathogen attachment project.

The paper is awaiting final comment from IFR.

The Produce WG agreed at its meeting last week that both this and the current draft of the second paper on the assessment of detachment (CFA/55/10) should be shared with Campden, Holchem and Steritrox to start to introduce common experimental approaches supported by CFA.

A third paper, which sets out a standard protocol designed to introduce consistency into produce washing e.g. hypochlorite dosing and pH control, has been also been developed (CFA/56/10) and is being shared by CFA with third parties.

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Secretary General

Produce Decontamination Assessment Protocol

Part 1 - Attachment

Abstract

Most, if not all, published efficacy assessments of produce decontamination agents do not appear to take into account the triphasic nature of bacterial contamination of produce, resulting in them either

a) not ensuring that inoculated bacteria are actually attached to the material to be decontaminated, or
b) ignoring the greatly enhanced robustness of attached organisms to decontamination by using suspension testing, which is inappropriate as it does not reflect true behaviour of attached organisms

Both of these approaches will result in exaggerated decontamination efficacies that are not apparent in reality.

The methodology described provides a robust approach to the determination of extent of decontamination of plant cell walls when subjected to a chemical or physical agent.

Aims

It is recognized that the use of biocides as decontaminants is not always successful (Adams et al. 1989; Garg et al. 1990; Brocklehurst 1994; Zhang and Farber 1996).

This protocol aims to provide a validated common approach for the assessment of produce decontamination when using washing systems, in line with criteria set out by EFSA (albeit in relation to the surface decontamination of products of animal origin), i.e. describing proof of the efficacy of the process under conditions where microorganisms are attached and/or have colonised plant material surfaces.

Use in research of a common validated protocol will enable true comparative efficacy to be judged.

This has been recognised by EFSA (2010), as set out in its requirements for technical dossiers on decontamination agents (for products of animal origin), which requires:

i) A comparison of surfaces of food of animal origin treated with the decontaminating agent and a corresponding untreated control food, where the only difference is the decontaminating agent and not the method of application or other factors e.g. if the decontaminating agent is used as a spray or dip, the control food must be sprayed or dipped under identical conditions.
ii) Tests on sufficient number of samples depending on the actual numbers and/or prevalence of the target organisms.

iii) The study design must be justified in relation to the specific claim(s) made for the substance and must include a consideration of statistical sound methodology and the capacity to measure expected changes with a probability of $P \leq 0.05$.

iv) The prevalence and/or the number of the target microorganisms must be measured before treatment, after treatment and at the end of the shelf life of the raw food to ensure that there is no repair of sublethally injured organisms.

v) The same testing should also be followed for the control foods. In addition, indicator microorganisms and total viable counts could be measured.

vi) Despite the antimicrobial treatment being intended to reduce the prevalence and/or numbers of pathogenic microorganisms, experimental data on the behaviour of non-pathogenic microorganisms such as indicator microorganisms and total viable counts, may assist judging the overall efficacy of the proposed treatment.

vii) The processing conditions in the study must be comparable with those where the decontaminating agent is intended to be used, e.g. in processing plant or a pilot plant.

viii) As far as possible the efficacy of the decontaminating agent should be tested on naturally contaminated foods.

ix) Particular attention should be paid to the sensitivity and specificity of the microbiological methods employed and to the adequacy and appropriateness of controls.

x) Experimental confirmation of the procedure to neutralise or eliminate the residual activity of the substance(s) to prevent interference with enumeration or detection of the target microorganism(s).

xi) Tests may be used with pathogenic bacteria to evaluate the efficacy, and should take account of strain diversity by testing at least 5-10 different strains, for example strains isolated from human cases and from the particular food. It is recommended to be able to differentiate between strains.

xii) Experimental demonstration that the concentration of the decontaminating agent proposed is justified, for instance laboratory data showing the effect of different concentrations of the antimicrobials on the target microorganism(s).

xiii) A description of the methods to control and monitor the concentration of the active substance(s) in the processing plant along operational time, including identifying factors that may influence the efficacy of the active substances (e.g. pH, temperature etc).

xiv) If the decontamination treatment effectively reduces numbers / prevalence of a pathogen and also extends the shelf-life, the impact of that extended shelf-life on survival and possible growth of other pathogens should be considered, e.g. *Listeria monocytogenes*.

The protocol described here focuses on the assessment of attachment prior to decontamination and therefore addresses all the points required to be covered by EFSA apart from viii), which is not possible for produce since contamination is so rare/random an event, and xiii), which is outside its scope, but which would be identified by application of the protocol in trials.

Introduction

The mechanism of *Salmonella* attachment to vegetable tissue is unknown. *Salmonella* Typhimurium has been shown to attach equally to both cut and intact surfaces of lettuce tissue (Takeuchi et al. 2000). Saggers et al (2008) demonstrated Salm. Typhimurium preferentially binds to material at the cell-wall junctions of intact potato tissue. It was also shown to attach to isolated cell wall material (CWM).

Previous unpublished work by the Institute of Food Research (IFR) led to the since-confirmed hypothesis that cells must be viable in order to attach to vegetable tissue (Saggers et al, 2008).

We regard bacterial colonization of prepared fruit and vegetable tissues to be the result of three phases:

1. an initial attachment phase,
2. a consolidation phase, which may involve the production of extracellular polymer, and
3. subsequent growth to form microcolonies.

Most studies of colonized vegetable tissue surfaces concentrate on the growth of bacteria on the product rather than on the initial attachment and consolidation phase. However, commercial decontamination processes are usually applied to prepared vegetable tissues within a few minutes of dicing, chopping or shredding, i.e. during the initial attachment and colonization phases. It is recognized that the use of biocides as decontaminants is not always successful.

Most, if not all, published efficacy assessments of produce decontamination agents do not appear to take into account this triphasic nature of bacterial contamination of produce, resulting in them either

a) not ensuring that inoculated bacteria are actually attached to the material to be decontaminated, or
b) ignoring the greatly enhanced robustness of attached organisms to decontamination by using suspension testing, which is inappropriate as it does not reflect true behaviour of attached organisms

Both of these approaches will result in exaggerated decontamination efficacies that are not apparent in reality.

It has already been shown that *Salmonella* serotype Typhimurium must be metabolically active to ensure attachment to plant cell walls and that when inactivated using heat, ethanol, formalin or Kanamycin after attachment Salm. Typhimurium remains attached to these sites (Saggers et al, 2008).

The following methodology, which was developed as part of that work, provides a robust approach to the determination of extent of decontamination of plant cell walls when subjected to a chemical or physical agent.

METHODOLOGY

Inocula

Inocula should be grown individually to stationary phase in 10 ml Trypticase Soy Broth (TSB, Oxoid) incubated at 25°C for 24 h and from this 100µl used to inoculate a fresh 10 ml TSB then incubated at either 20°C for 24h or 15°C for 27h (i.e. until stationary phase). Bacterial cultures should be subsequently diluted in peptone salt dilution fluid to the required cell density.

Unpublished work (IFR) shows that the temperature history of the inoculum can have an effect on attachment with prior exposure to low temperatures reducing the level of attachment, hence the choice of these conditions. For example if it is intended to establish attachment of organisms originating from a chilled environment then the organism needs to be grown to stationary phase in a chilled environment and attached at chill also.

Inoculation

Inoculation of individual pieces or fresh-cut plant tissue

Plant tissue samples should be immersed in the inoculum (prepared as described above) and incubated at the required temperature in sterile disposable plastic Petri dishes (90mm diameter, 4 discs in each Petri dish, 20mls of inoculum). At 30 minutes tissue samples were carefully removed from the Petri dish and each tissues sample rinsed in peptone salt dilution fluid (100 ml) with stirring for 1 minute in order to remove any associated but unattached bacteria.

Previous research (Garrood *et al* 2004, Saggers *et al*, unpublished, T. Brocklehurst personal communication) has shown that the attachment phase is complete, i.e. maximal level of attachment, at 30 minutes after inoculation. This has been established for a number of key pathogenic bacteria, including *Salmonella* and, *Listeria monocytogenes*, and spoilage bacteria on a number of fruit and vegetable tissues (potato, carrot, green pepper and melon).

Inoculation of entire tissues i.e. whole head lettuce

Whole heads of lettuce were inoculated with bacteria. The inoculated heads could then be processed as per factory processing and decontamination methods tested. For whole head inoculation, a whole head of lettuce had the butt end and outer leaves removed before immersion in the inoculum for 30 minutes. After 30 minutes the lettuce was drained for 30 minutes to remove excess inoculum. This method results in maximal attachment of bacteria to whole head tissues (E. Saggers, personal communication.)

Incubation

Inoculated tissue should be incubated preferably at ambient and no lower than 5°C for 24 hours before enumeration to allow sufficient time for the production of extracellular polysaccharide to ensure established attachment.

Enumeration of attached bacteria

Bacteria remaining on the tissues sample are then removed by blending with 10mls peptone salt dilution fluid for 120 seconds using a Stomacher Lab-blender. The number of viable bacteria in the resultant suspension is determined by spreading 50 μl of this suspension, or dilutions of it made in peptone salt dilution fluid, onto the surface of plates of Oxford Agar (*L. monocytogenes*) or PCA (all other organisms) using the Spiral Plate Maker. Plates are incubated at 35°C for 24 hours.

Confirmation of attachment through this method

Confirmation of attachment *via* this method was provided in two separate studies using a *Gfp*-producing fluorescent strain of *S.*serovar Typhimurium (*S*. serovar Typhimurium JH3016).

Firstly cut disks of potato tissue were inoculated following the procedure outlined above and the tissues imaged using a confocal scanning laser microscope (CSLM). The resultant image (Figure 1) show *Salmonella* clearly attached in large numbers to the potato tissue.

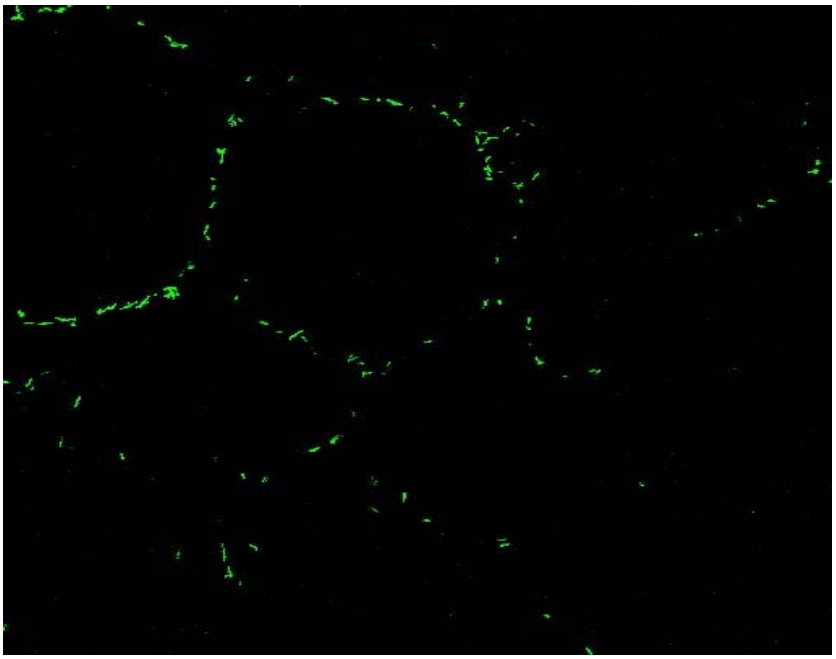

Figure 1: Confirmation of attachment of *Salmonella* to cut potato tissue: CSLM image of *Salmonella* attached to the surface of potato tissue. Green = Individual bacterium of *S*. serovar Typhimurium JH3016

In addition the attachment of *Salmonella* serovar Typhimurium JH3016 to potato CWM was confirmed by flow cytometry using the method described by Saggers *et al* (2008). A hundred microlitres of viable *S.* serovar Typhimurium JH3016 was added to re-hydrated CWM and incubated at 20°C, 120 rev min^{-1} for 30 min to allow attachment. After this time, samples were washed to remove unattached bacteria. The CWM was then collected into a stomacher bag and

stomached (Seward 80 Biomaster; Fisher Scientific, Loughborough, UK) for 60 s to remove the attached bacteria from the CWM. The sample was washed to remove any residual CWM and the number of bacteria in the filtrate, and therefore previously attached to the CWM, was enumerated by flow cytometry. For this, the sample was fixed in 4% (v/v) formalin for 2 min, the filtrate was centrifuged at 15 000 g for 5 min and then washed twice by centrifugation with PBS. Samples were then immediately analysed by flow cytometry. This used a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a 15-mW air-cooled argon ion laser as the excitation light source (488 nm). In order to perform absolute counts, samples were mixed with a known number of FluoSpheres_ carboxylate-modified crimson fluorescent microspheres (Molecular probes F-8816; Invitrogen, Paisley, UK). The beads were detected on a separate channel from the constitutively *Gfp*+- expressing *S.* serovar Typhimurium strain JH3016. All parameters were collected by using amplification gains set on LOG mode.

Acquisition was stopped when 10 000 beads were counted, allowing the volume of sample used to be determined. The number of *Gfp*+-expressing *Salmonella* cells detected in that same volume was subsequently calculated after analysis with CellQuest 3Æ3 software (Becton Dickinson) and converted into number of *Salmonella* cells per millilitre. Control samples of filtered PBS and uninoculated CWM were also analysed to establish background noise within the samples.

To validate the absolute counts obtained from calculations of bead and *Gfp*+ fluorescence, a viable count was performed on the sample before fixation for flow cytometry. Fifty microlitres of the sample were inoculated onto the surface of duplicate plates of PCA using a Spiral Plate Maker (Don Whitley Scientific, Shipley, UK). PCA plates were incubated at 30°C for 24 hours before enumeration.

Validation across different materials

The attachment assessment protocol has been validated for *Salmonella* serovar Typhimurium using cell wall material extracted from green pepper, melon and potato. The kinetics of attachment are mirrored with lettuce although air spaces within the leaf can accommodate bacteria if the temperature differential of the washwater vs leaf is not managed appropriately, i.e. the leaf should no warmer than the washwater in order to negate incursion into air spaces.

Summary

This protocol provides a validated common approach for the first step in the assessment of produce decontamination pathogen attachment to plant surfaces to enable true comparative efficacy of decontamination processes to be judged.

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