Detection and enumeration of viable *Listeria monocytogenes* cells from ready-to-eat and processed vegetable foods by culture and DVC-FISH.

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Listeria monocytogenes is an important agent of foodborne disease, showing low prevalence but high mortality. There is evidence that vegetables are important vehicles of transmission, especially those minimally processed (fresh, fresh-cut vegetables under modified atmosphere packaging (MAP) or frozen) and directly exposed to consumers. The aim of this work was to detect and enumerate viable *Listeria monocytogenes* cells by culture and molecular methods from vegetable foods.

A total of 191 vegetable samples (fresh, frozen and fresh-cut under modified atmosphere packaging) were studied. *L. monocytogenes* was detected and identified by selective plating, PCR and DVC-FISH.

An isolation rate of 4.19% was obtained by culture, with a higher incidence in frozen vegetables. Six isolates belonged to serotype 1/2a and two to 4b. Counts were below <100 cfu / g for the eight positive samples, according to the food safety criteria established for the RTE by Commission Regulation EC N° 1441/2007.

Multiplex PCR method yielded a greater number of positive samples (10.47%). DVC-FISH technique determined that viable cells of *Listeria monocytogenes* were present in 32.98% of the samples, containing up to 4.97 log 10 viable cells/g.

1. Introduction

Listeria monocytogenes is the causal agent of several worldwide foodborne diseases, showing low prevalence but high mortality. *Listeria* species are present in a variety of environments, including soil, water, effluents and foods. With manufactured

ready-to-eat foods being consumed in increasing quantities, it is not surprising that *L. monocytogenes* has become recognized as an important opportunistic human foodborne pathogen (Liu, 2006). Freeze injury reversibility of *L. monocytogenes* transforms contaminated frozen foods into a potential source of infection (Flanders & Donnelly, 1994). Some authors have also suggested that some strains could be specially adapted to colonize the processing plant equipments, thus persisting for long periods of time (Pappelbaum *et al.*, 2008).

There is evidence that vegetables are important vehicles of transmission, especially those minimally processed (fresh, fresh-cut vegetables under modified atmosphere packaging (MAP) or frozen) and directly exposed to consumers. Many works have focused on the detection of *Listeria monocytogenes* in ready-to-eat (RTE) food. The organism has been found in cabbage, celery, carrot, lettuce, cucumber, onion, cabbage, potatoes, tomato and fennel (Beuchat, 1996). Recently, a study concluded that refrigerated ready-to-eat meals with extended shelf life are high risk products for containing *Listeria monocytogenes* (Uyttendaele *et al.*, 2009). Because of that, safety of minimally processed ready-to-eat foods must be tested in order to ensure the absence of any risk. There is a European regulation that establishes a value lower than 100 cfu/g of *L. monocytogenes* for RTE foods able and unable to support the growth of this pathogen other than those intended for infants and for special medical purposes (Commission Regulation (EC) No 1441/2007).

The concept of viable but non-culturable (VBNC) physiological state of bacteria has been applied to a large number of enteric bacteria such as *Salmonella enteritidis*, *Escherichia coli*, *Vibrio cholerae* or *Campylobacter jejuni*, to indicate an inability for their replication in certain stress situations, as a survival strategy (Rollins & Colwell, 1986). These VBNC forms, under adequate environmental conditions, could be transformed again into a culturable pathogen (Wong & Wang, 2004). The main

problem caused by the presence of the VBNC state is the impossibility to detect these bacteria by culture, which could result in an underestimation of its presence in foods (Besnard, Federighi, Declerq & Cappelier, 2000(a)).

Listeria monocytogenes can enter the VBNC state under environmental stress conditions such as low nutrient source, extreme temperatures or high osmotic concentration of the medium (Besnard, Federighi, Declerq, Jugiau & Cappelier, 2002). A recent study tested the ability of the bacteria to recover its culturability after entering the VBNC state. It was observed that it mainly depends on the relative humidity of the environment: in dry environments, a recovery was not observed. However, when relative humidity was close to 100%, *Listeria* was able to grow on conventional culture media (Dreux *et al.*, 2007).

Traditional culture methodology for detecting *L. monocytogenes* may require about 4-5 days. An alternative to conventional detection methods for foodborne pathogens are molecular techniques such as PCR.

Ribosomal rRNA probe hybridization without culturing (Fluorescent *in situ* Hybridization, FISH) has become widely adopted for detection of specific bacterial groups in mixed populations (Wagner, Horn & Daims, 2003). The FISH assay is less sensitive to inhibitory substances than PCR and has shown to be a very useful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology (Bottari, Ercolini, Gatti & Neviani*,* 2006). It has been successfully used for detection and identification of different pathogens in foods, surface water, drinking water and wastewater, (Moreno, Piqueres, Ferrús & Alonso*,* 2007; Piqueres, Moreno, Alonso & Ferrús, 2006; Garcia-Armisen & Servais, 2004).

A limitation of FISH and PCR techniques is that they are not able to discriminate between dead and viable cells (Okoh, Odjadjare, Igbinosa & Osode 2007), what can

lead to false positive results. Although the use of enrichment selective media promotes the growth of viable cells, dead cells can still be present. The Direct Viable Count (DVC) procedure (Kogure, Simidu & Taga, 1979) involves exposing bacterial cells to a medium that contains antibiotics that prevent cellular division, by inhibiting DNA-gyrase. The incubation of bacteria in this conditions increases the amount of ribosomal RNA in the cell and promotes elongation of metabolically active cells (Doudu & Colquhoun, 2010), which are then enumerated as viable cells (Servais, Prats, Passerat & Garcia-Armisen, 2009). Therefore, the combination of DVC with FISH has been proposed as a very useful tool for monitoring viable cells in different environments (Baudart, Coallier, Laurent & Prévost, 2002; Piqueres, Moreno, Alonso & Ferrús, 2006).

The aim of this work was to study the presence of *Listeria monocytogenes* in vegetable samples available to consumers in markets and supermarkets from Valencia (Spain). This pathogen was detected by culture according to standard methods and by PCR. Enumeration of viable cells was also performed by DVC-FISH method. In addition, isolates were serotyped, and the presence of lmo2821 virulence gene was investigated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two reference *L. monocytogenes* strains (CECT 4032 and CECT 936, Colección Española de Cultivos Tipo, Spain) were used. They were grown on TSA (Casein – peptone soy meal - peptone agar for microbiology, Merck, Darmstadt, Germany) for 24 h at 37 ºC and subcultured overnight in Brain-Heart-Infusion (BHI) broth (Merck, Darmstadt, Germany) for all the assays.

2.2. Sampling

A total of 191 samples of RTE foods of vegetal origin were collected from supermarkets in different presentation formats: fresh, frozen and fresh-cut vegetables under MAP (Table1). Samples were processed before three hours after arrival at the laboratory in sterile conditions.

2.3. Detection and enumeration of *L. monocytogenes* by direct plating

The detection and enumeration of *L. monocytogenes* were carried out as described in the ISO 11290-1:1996 and ISO 11290-2:1998 protocols, respectively. Briefly, 25 g of each sample were homogenized with 225 ml of half-concentrated selective supplement of sterile Fraser broth base (primary enrichment) (Scharlau Chemie S.A., Barcelona, Spain) in an Homogenizer Lab-Blender 400 (Seward Medical, London, UK) for 2 min and incubated at 30 °C for 24 h. Afterwards, 0.1 ml of the primary enrichment was inoculated in 10 ml of Fraser broth supplemented with Fraser selective supplement (secondary enrichment) (Scharlau) and incubated at 37 °C for 24 h. Cultures were streaked onto Palcam (Scharlau) and ALOA (Scharlau) plates and incubated at 37 °C for 24 h. Typical colonies were selected and submitted to Gram staining, catalase and oxidase test. Hemolytic activity on sheep blood agar (Columbia agar supplemented with 5% of defibrinated horse blood, Scharlau Chemie S.A., Barcelona, Spain) was also performed.

Biochemical characterization of all the isolates was performed using the API *Listeria* identification system (Biomérieux, Marcy l'Etoile, France).

6

2.4. Characterization of *L. monocytogenes* isolated strains

Serotyping of *L. monocytogenes* isolates was carried out by the agglutination method, using commercial *Listeria* antisera kit (Denka Seiken.Co, Ltd., Tokio, Japan), OI–OII, OV–OVI, OI, OIV, OVI, OVII, OVIII, OIX for somatic antigens, according to manufacturers. Detection of flagellar antigens H-A, H-AB, H-C, H-D was performed according to Ueda *et al.* (2002).

A PCR for amplification of the gene encoding lmo 2821 internalin protein J (Sabet, Lecuit, Cabanes, Cossart & Bierne, 2005; Liu, 2006) was performed, using the primers described by Liu, Ainsworth, Austin & Lawrence (2003) which amplify a fragment of 611pb of the gene. Strain CECT 4032, that has often been associated with cases of meningitis was used as a positive control.

2.5. Detection and enumeration of *L. monocytogenes* by molecular methods

2.5.1. Multiplex PCR

During the development of the ISO 11290-1:1996 method for the detection of *Listeria monocytogenes*, aliquots were taken before the primary enrichment incubation and after secondary enrichment. Cells were harvested after being centrifuged at 14000 x g for 30 s. They were washed twice with sterile phosphate buffer and then suspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). For DNA isolation the Realpure genomic DNA isolation kit (RBMEG02, Durviz, Paterna, Spain) was used, following the manufacturer's instructions. Extracted DNA was stored at -20ºC.

Multiplex PCR was applied for *L. monocytogenes* detection using primers described by Bansal, McDonell, Smith, Arnold & Ibrahim (1996), which amplify a genus specific 938 bp fragment of 16S rDNA of *Listeria* sp. and a species specific 750 bp fragment of *hly*A gene of *L. monocytogenes*. A final reaction volume of 30 µl was obtained by

addition 3 µl of sample, 20 pmol of each primer, 0.2 mM of each deoxynucleotide, 2.5 mM MgCl$_2$ and 2 U of *Taq* polymerase (New England Biolabs, U.K.). For amplification, there was an initial DNA denaturing step at 95 ºC for 1 min, followed by 40-cycle reaction (94 ºC for 30 s, 51ºC for 20 s an 74ºC for 20 s). A final extension step was undertaken at 72 ºC for 2 min (Zamora, Ossa, Carrascal, Poutou & Jimenez, 2000). Electrophoresis at 100 V for 1 h through 1% (wt/vol) SeaKem LE agarose gel (FMC Bioproducts, Denmark) was applied for analyzing PCR products. Amplimers were visualized by staining with ethidium bromide under UV light. A 100 bp DNA ladder was used as a molecular weight marker.

2.5.2. Detection and enumeration of *L. monocytogenes* by DVC-FISH

An oligonucleotide probe complementary to a 16S rRNA region of *Listeria monocytogenes* was used (*Lmon* probe: 5´-CTATCCATTGTAGCACGTG-3´, Moreno, Ballesteros, García-Hernández, Santiago, Gonzalez. & Ferrús, 2011). The probe targets position 1242 to 1260 in *L. monocytogenes* 16S rRNA. The two *Listeria* reference strains included in this study were used as positive controls. The probe was synthesized and labelled by MGW Biotech (Mannheim, Germany) with 5 (6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) and CY3.

For FISH analysis, a volume of 1 mL of each sample was centrifuged (8000 rpm, at 4ºC for 10 min). Pellet was resuspended in PBS buffer (130 mM sodium chloride, 10 mM sodium phosphate, [pH 7.2]), and then fixed with an ethanol/PBS (50:50) mixture (Amann, Ludwig & Schleifer, 1995). Fixed samples were stored at -20ºC until their hybridization.

Ten µL of each fixed sample were placed on a gelatin-coated slide, allowed to air-dry, treated with lysozyme and dehydrated (50, 80, 100% ethanol) as previously described (Wagner *et al.*, 1998). A final concentration of 20% formamide in the

hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.6) was enough to reach the specificity of the whole cell hybridization. The EUB338 universal probes mixture, complementary to a region of 16S rRNA of the domain *Bacteria* (Amann, Ludwig & Schleifer, 1995) was used as a positive control. The slides were then mounted with FluoroGuard Antifade Reagent (Bio-Rad, Madrid, Spain) and visualized by epifluorescence Olympus microscopy BX50 with 460-490 nm (U-MWIB) and 520-550 nm (U-MWIG) exciter filters.

For DVC procedure, 50 ml aliquots were taken from homogenized vegetable samples previously to enrichment. Samples were then centrifuged at 4200 rpm for 15 min. The pellet was resuspended in 1 ml phosphate buffer 1X PBS and added to 9 mL of BHI broth supplemented with yeast extract (2.5 mg/mL) and 2 mg/mL of the antimicrobial agent Ciprofloxacin (SIGMA Chemical Co., St. Louis, Mo.), as described by Besnard, Federighi & Cappelier (2000(b)). DVC broths were incubated at 37 ºC in aerobic conditions and aliquots from each dilution were taken after 7h and processed for FISH as described above. In accordance with other authors (Regnault, Martin-Delautre & Grimont, 2000; Kogure, Simidu & Taga,, 1979), cells were estimated as viable when their elongation was at least twice their original length.

3. Results

3.1. Prevalence and enumeration of *L. monocytogenes* in vegetable foods

L. monocytogenes was isolated from eight products (4.19% of the examined samples; Table 2). Strains were obtained from 8.33% of frozen food (two spinach, one broccoli and one vegetable stir-fry samples), 4.28% of fresh-cut vegetables under MAP (one isolate from spinach and two from broccoli), and from a sample of fresh spinach (1.37% of fresh samples). Half of the *L. monocytogenes* isolates were

9

obtained from spinach in different commercial presentations: two in frozen product, one in fresh and, finally, one in a fresh-cut product under MAP.

The counts of *L. monocytogenes* in the positive samples were <100 cfu/g in all cases.

3.2. Characterization of isolates from vegetable samples

The eight obtained isolates were identified as *L. monocytogenes* by the API system and by mPCR. Six strains (75% of the isolates coming from 1fresh-cut spinach under MAP, 2 frozen spinach, 2 samples of fresh-cut broccoli under MAP and one frozen broccoli) belonged to serotype 1/2a and two of them (25%), one from frozen stir-fry and the other from fresh spinach, to serotype 4b.

After specific PCR, all the *L. monocytogenes* isolates showed the presence of the 611 bp band corresponding to a fragment of the *lmo* 2821 gene (Figure 1).

3.3. Detection and enumeration of *L. monocytogenes* by molecular methods

Twenty samples analyzed by multiplex PCR after the secondary enrichment were positive for *L. monocytogenes* presence (10.47%). Frozen spinach was the food that yielded the greatest number of positives (Table 2). All samples were negative prior to enrichment.

When DVC technique was applied, sixty three samples (32.98%) showed elongated viable *L. monocytogenes* cells (Table 2 and Figure 2).

Spinach was the food where the higher number of viable cells was detected. In total, detection was possible in 26 samples. Counts between 2.85 and 4.97 log10 viable cells/g of food were obtained. DVC-FISH positive results were obtained in six

samples of parsley, three fresh and three frozen, with values between 3.46 and 3.85 log10 viable cells/g of food. Viable cells of *Listeria monocytogenes* were observed in eight samples of broccoli, five in the frozen format and three in fresh-cut vegetables under MAP, with viable counts ranging from 2.9 to 4.92 log10 cells/g of food. Five samples of frozen stir-fry were positive, with counts between 3.2 and 4.18 log10 viable cells/g of food. Four samples of fresh-cut sprouts showed counts ranging from 3.16 to 3.46 log10 viable cells/g of food. Nine lettuce samples were also positive, with counts between 2.85 and 3.55 log10 viable cells/g of food. The only positive sample of Four Seasons salad yielded a high number of viable cells, with a value of 4.35 log10 cells/g of food.

4. Discussion

The presence of *L. monocytogenes* on many types of raw and minimally processed vegetables addressed to human consumption has been clearly demonstrated in many countries. Moreover, listeriosis cases are increasing at a global level, due in many cases to cross-contamination of processed foods (Wilks, Michels & Keevil, 2006). For manufacturers and retailers of packaged fresh vegetables, this is a cause of concern because of *L. monocytogenes* prevalence in the environment and its ability to grow at refrigeration temperatures (Beuchat, 1996).Therefore, adequate controls and hygienic manipulations must be established.

In this work we have detected and identified *L. monocytogenes* from different vegetable foods purchased in shops located in the city of Valencia (Spain), showing the existing risk for the consumers of RTE. Initially, the method described by current regulation UNE-EN ISO 11290-1:1997/A-1: 2005 and UNE-EN ISO 11290-2:2000/A-1: 2005 was applied. The eight presumptive *L. monocytogenes* isolated colonies

were identified by confirmatory tests (catalase, oxidase, β-hemolysis and subsequent confirmation by API-Listeria). As biochemical identification might be unstable in some cases (Kühn, Brauner & Möllby, 1990), isolates were confirmed by *L. monocytogenes* specific multiplex PCR*.*

The isolation rate was identical for the two culture media used. An isolation percentage of 4.18% (8/191) was obtained, what agrees with other studies conducted in Spain, where L*. monocytogenes* was isolated in 8 out of 103 samples (7.80%) of raw vegetables (Simon, Tarrago & Ferrer, 1992). In a recent german study (Schwaiger, Helmke, Holzel & Bauer, 2011), in 1001 investigated vegetables, only four *L. monocytogenes* were found. In Italy, L*. monocytogenes* was isolated from a total of 7/102 (6.90%) samples of different raw vegetables (Gola, Previdi, Mutti & Belloi, 1990) and in Ireland in 3/21 (14.30%) of packed under MAP fresh-cut salads ready for consumption with ingredients such as cabbage, carrots and lettuce (Harvey & Gilmour, 1993).

Four *L. monocytogenes* strains were isolated from spinach (two in the frozen presentation, one in the fresh-cut product under MAP and one in fresh format), three from broccoli (one from frozen food and two from fresh-cut product) and one from frozen stir fry. In our work, counts were below <100 cfu / g for the eight positive samples, according to the food safety criteria established for the RTE by Commission Regulation EC N° 1441/2007.

Due to the limitations of traditional methods for the detection of fastidious pathogens and the need of a later identification, what leads to a delay in obtaining results, numerous alternative detection methods have been developed, most of them based on the analysis of nucleic acids. Among them, we have selected multiplex PCR for *L. monocytogenes* detection, and DVC-FISH for specific detection of viable cells, because of specificity and speed of both methods. In this work, as expected, higher

numbers of positive samples for *L. monocytogenes* were obtained using the PCR and DVC-FISH technique better than the culture method.

The detection of *L. monocytogenes* by PCR was only possible by including an enrichment step, which could be due to an increase in viable cells (Lee & McClain, 1986) or to the dilution of potential inhibitors present in the sample (Gasanov, Hughes & Hansbro, 2005). The most common inhibitors are organic and phenolic compounds, glycogen, lipids, humic compounds and heavy metals (Wilson, 1997), some of them present in the soil for cultivation (fertilizer) and in the crop. By multiplex PCR, 20 positive samples of *Listeria monocytogenes* were detected. However, possible false positives due to the presence of bacterial DNA from dead or non-viable cells cannot be discarded because a limitation of FISH and PCR techniques is that they are not able to discriminate between dead and viable cells (Okoh *et a*l., 2007), what can lead to false positive results (Moreno, Ballesteros, García-Hernández, Santiago, Gonzalez. & Ferrús, 2011)

So we proceeded to the detection of viable cells by DVC-FISH. The detection of pathogens by FISH has been widely used because of its specificity, speed and insensitivity to false positives (Moreno, Botella, Alonso, Ferrús, Hernández & Hernández, 2003). Viable cells of *L. monocytogenes* were detected in 63 of the 191 tested samples (32.98% of selected foods), including possible VBNC cells that would also be potentially infectious (Besnard, Federighi, Declerq, Jugiau & Cappelier 2000(b)). In some analyzed vegetables, up to 10^4 viable cells/g of *L. monocytogenes* were detected without an enrichment step, thus showing their importance of fresh vegetables as a vehicle for transmission to humans. It must be considered that the DVC-FISH technique does not require prior enrichment for detecting the presence of viable cells of the pathogen in samples with an initial contamination above 10^2 viable

cells/g. In addition, the time required for the detection and identification of potential pathogens by FISH is considerably reduced since it requires no further identification.

All strains of *L. monocytogenes* are potentially pathogenic, but their virulence may vary. Many authors have studied the target proteins and genes in virulent and avirulent strains in order to establish differences in *L. monocytogenes* pathogenesis. lmo 2821 gene product (internalin J) seems to be present in all virulent strains of *L. monocytogenes* (Liu, 2006). In all the isolates identified in this study, the band of 611 bp, resulting from the amplification of a fragment of lmo 2821 gene was observed (Figure 1).

From the 8 isolates, 6 belonged to serotype 1/2a and the remaining two were identified as serotype 4b. Both are the most common serotypes isolated in human listeriosis cases (Liu, 2006) and have been previously detected in foods (Vitas, Aguado & García-Jalón, 2004; Kiss, Tirczka, Szita, Bernath & Csiko, 2006).

In this study the presence of *Listeria monocytogenes* was detected in a higher percentage of frozen samples than in other product presentations, especially in spinach. This could be due to the capability of this pathogen to resist freezing and cooling temperatures (Garrido, Torroba, García-Jalón & Vitas, 2008), remaining in the sample while the rest of microbiota is reduced. However, fresh vegetables are those involving a greater risk for consumers since they do not require any pretreatment before being consumed. According to our results, risk of *L. monocytogenes* in RTE vegetable foods cannot be discarded, and it should be recommended that products are subjected to a disinfection treatment, such as washing with chlorinated products, before its consumption.

Aknowledgments

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Table 1. Food vegetables analyzed.

FOOD	PRESENTATION			TOTAL
	FRESH	FRESH-CUT (MAP)	FROZEN	
Spinach	18	23	16	57
Parsley	11	11	15	37
Broccoli	17	16	6	39
4 Seasons salad	0	12	0	12
Sprouts	0	8	0	8
Lettuce	27	0	0	27
Vegetable stew	0	0	6	6
Stir fry	0	0	5	5
TOTAL	73	70	48	191

Table 2. *Listeria monocytogenes* detection results and counts of viable cells by DVC-FISH.

Presentation	Type of product	Total Samples	Isolation (%) positives	Multiplex PCR positives (%)	DVC-FISH Number of positive samples (%)	DVC-FISH counts ($\log_{10}$ ufc/g)
FRESH	Spinach	18	1	2	5	4.72-4.97
	Parsley	11	-	-	3	3.46-3.71
	Broccoli	17	-	2	-	
	Lettuce	27	-	1	9	2.85-3.55
	Total	**73**	**1 (1.37%)**	**5 (6.85%)**	**17 (23.29%)**	
FRESH-CUT (MAP)	Spinach	23	1	3	7	2.85-3.43
	Parsley	11	-	-	-	
	Broccoli	16	2	1	3	2.9-3.15
	4 Seasons salad	12	-	1	1	4.35
	Sprouts	8	-	-	4	3.16-3.46
	Total	**70**	**3 (4.28%)**	**5 (7.14%)**	**15 (14.29%)**	
FROZEN	Spinach	16	2	7	14	3.16-4.15
	Parsley	15	-	2	3	3.7-3.85
	Broccoli	6	1	1	5	4.41-4.92
	Vegetable stew	6	-	-	-	
	Stir fry	5	1	-	5	3.2-4.18
	Total	**48**	**4 (8.33%)**	**10 (20.83%)**	**27 (56.25%)**	
TOTAL		**191**	**8 (4.19%)**	**20 (10.47%)**	**63 (32.98%)**	

21

Figure 1. PCR detection of *lmo* gene in *L. monocytogenes* isolated from vegetables samples. (Lines 1-4; 6-9) showing the specific 611 bp fragment of *lmon* 2821. Line 5: 100 bp ladder . Line 9: positive control. Line 10: negative control.
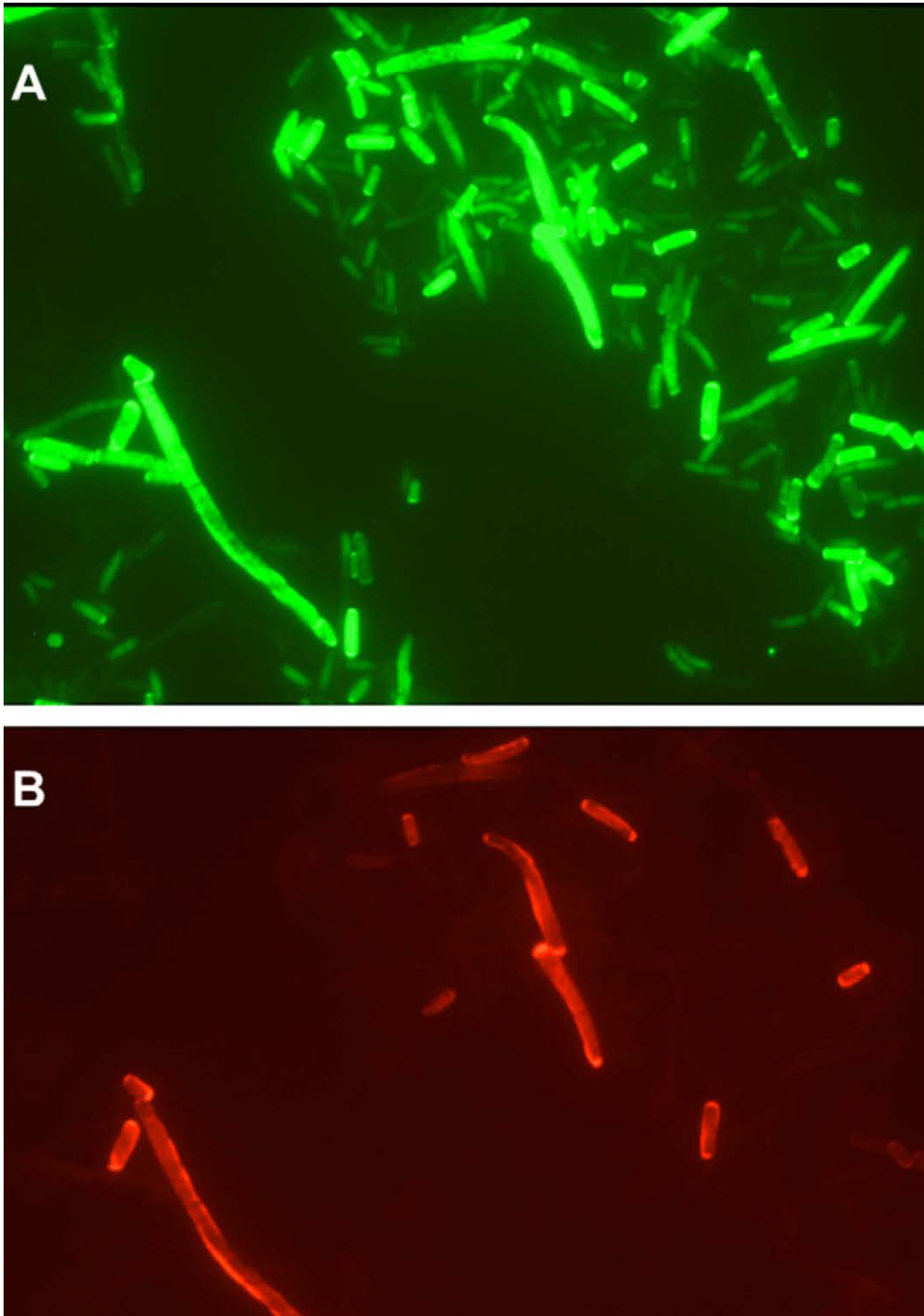
Figure 2. FISH identification of viable (elongated) *L. monocytogenes* in frozen spinach after DVC treatment. (A) EUB338-FLUOS probe (B) *Lmon*-CY3. Arrow shows an elongated viable *L. monocytogenes* cell.

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