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Evaluation of the MicroFoss system for the detection of *Listeria* species in environmental samples

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Abstract

The MicroFoss system was evaluated for its ability to detect *Listeria* species in environmental samples. The sensitivity and specificity of the MicroFoss were determined in relation to a standard culture method for *Listeria* detection. The sensitivities of both the MicroFoss and standard culture methods were similar (88.4%—MicroFoss, 90.7%—Culture) based on the total number of positive results obtained by both methods. The MicroFoss system detected *Listeria* spp. in 12 samples, which were not detected by culture, and the culture method detected *Listeria* spp. in 15 samples, which were not detected by the MicroFoss method. This was likely due to uneven distribution of low levels of *Listeria* organisms in the split sponge samples used to assess the performance of these test methods. The specificity value determined for the MicroFoss system was 92.7%. The majority of microbes causing false positive results in the MicroFoss system were *Bacillus* species, which were readily distinguishable from *Listeria* species by a simple Gram stain and morphological features. *Listeria monocytogenes* (89.4%—MicroFoss, 88.0%—Culture) and *Listeria innocua* (8.8%—MicroFoss, 7.7%—Culture) were the most common isolates of *Listeria* detected by the two test methods, with *L. monocytogenes* being the most predominant isolate detected. The highly comparable results and rapid nature of the MicroFoss system demonstrate its effectiveness as a detection system for species of *Listeria* in environmental samples. The fact that the sensitivity of the MicroFoss system was similar to that of the culture method and the *Listeria* results were obtained within 48 h of testing, support the use of the MicroFoss as an alternative rapid method for screening large numbers of environmental samples for *Listeria* spp.

Keywords: MicroFoss; *Listeria*; Standard culture method; Environmental samples

1. Introduction

The ubiquitous and pathogenic properties of *Listeria monocytogenes* has led to numerous studies focussing on the rapid detection of *L. monocytogenes* in foods as well as measures to prevent contamination of food products during and after processing by this organism. *L. monocytogenes* causes listeriosis in humans, especially in children, the elderly and the immuno-compromised. Listeriosis may only take the form of flu-like symptoms; however, there is also the possibility of meningitis, encephalitis or septicemia, with high mortality rates (Murray et al., 1995).

The contamination of food products with *Listeria* spp. is thought to be mainly due to post-processing

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contamination, which in turn may reflect the environmental hygiene of food production areas (Bell and Kyriakides, 1998). In fact, areas of poor sanitation in a plant may enable *Listeria* spp. to form biofilms, which may allow them to persist in the plant environment and increase the probability of food contamination (Blackman and Frank, 1996; Wong, 1998). In order to prevent *Listeria* outbreaks, environmental testing for *Listeria* species is a key component of any food processing plant's testing regime. Food processing plants that carry out environmental testing for *Listeria* spp. for their Quality Assurance (QA) and Hazards Analysis Critical Control Point (HACCP) programs require accurate and rapid methods for the detection of these organisms in order to avoid delays in releasing perishable food products for sale.

The MicroFoss (BioSys) system is an instrument that has the potential to rapidly test a large number of environmental samples for the presence of *Listeria* spp. within 30–48 h of testing. The MicroFoss system's technology is based on colorimetric detection of microbial growth. With respect to *Listeria* detection, esculin is hydrolyzed by the enzyme esculinase to esculetin, which in turn reacts with ferric ions causing a darkening of the medium (Peng and Shelef, 2001). Optical measurements are taken in a semi-fluid area, whereby the presence of an agar plug at the bottom of each test vial allows passage of small molecules and ions only. The agar plug ensures that interference from food particles is avoided. Changes in optical units are recorded and used to determine the detection time for a specified microbe.

The purpose of this study was to evaluate the application of the MicroFoss system for the detection of *Listeria* spp. in environmental samples in comparison to the standard *Listeria* culture method.

2. Materials and methods

2.1. Sample collection

Environmental samples were obtained from a food processing environment using environmental sponge kits (BioSys, Ann Arbor, MI) containing sponges premoistened with neutralizing buffer, sterile gloves and sample bags. A total of 552 environmental sponge samples were collected and tested using the MicroFoss system and *Listeria* culture methods during the duration of this study. A biased sampling technique was employed for the first 392 samples, such that areas that were more likely to harbour *Listeria* spp. (non-food contact surfaces such as drains and floors) were sampled more frequently than other areas (food contact surfaces such as equipment surfaces). The remaining 160 samples were sampled using a non-biased sampling technique. A 10-cm^2 area of each surface was swabbed (in four directions within the 10-cm^2 area) using the sterile gloves, sterile sponges and sample bags provided. Between 14 and 20 sponge samples from the participating food processing plant were shipped to the lab twice per week for a total of 24 weeks. The samples were shipped in Styrofoam coolers for delivery to the lab within 24 h of sample collection.

2.2. Sample preparation

Samples were received and prepared within 24 h of sample collection. Upon arrival, sponge samples were aseptically transferred to sterile stomacher bags, to which 25 ml of sterile 0.1% peptone water was added per bag. The samples were then stomached (Seward Model 400) for 2 min and two 10 ml aliquots were transferred into a Whirlpak™ bag and a sterile stomacher bag. Spiked sponge samples were prepared in order to determine the detection limit of the MicroFoss system for *Listeria* spp. *Listeria* species tested included *L. monocytogenes*, *Listeria innocua*, *Listeria welshmeri*, and *Listeria ivanovii* at inoculum levels of 1, 10 and 100 cfu/ml.

2.3. MicroFoss Listeria test method

A 40-ml volume of Pre-Enrichment (PE) medium (BioSys) was aseptically added to each WhirlPak™ bag containing the sample suspension. In order to minimize air exposure, each of the Whirlpak™ bags was rolled down to the level of the broth. The bags were then placed in a 35 °C incubator for a pre-incubation period of 6.5–7 h. Prior to the end of the pre-incubation period, Listeria selective supplement (BioSys), in the form of lyophilized pellets, were individually re-hydrated with 2.2 ml of sterile water.

Each vial containing 5 ml of Listeria medium (specific to sponge sampling, BioSys) had 200 μl of the rehydrated supplement aseptically added. Following the pre-incubation period, each of the sample bags were mixed well by inverting the bags end to end 25 times by hand. Five milliliters of each of the incubated broths were transferred to the appropriate Listeria vials containing the supplement. Notably, sample bags that displayed a blackening of the medium were diluted, such that 0.5 ml of the sample was added to the vial and an additional 4.5 ml of PE medium was also added. Both the diluted (0.5 ml sample volume) and non-diluted (5 ml sample volume) were prepared for analysis.

For control purposes, two vials were separately inoculated with *L. monocytogenes* (ATCC 19115) at a concentration of 10^2 cfu/ml, and *Bacillus cereus* (B52, BioSys lab strain) at a concentration of 10^3 cfu/ml. Also, one vial containing the Listeria medium and supplement was used as a blank control. A set of three control vials were run with each batch of samples tested.

All of the inoculated vials were gently inverted 10 times. The vial caps were slightly loosened and the vials were then placed in the MicroFoss instrument and tested for 30–48 h at 35 °C (BioSys, 2001). Samples that had not achieved a detection time within the 30-h time period remained in the MicroFoss instrument for up to an additional 18 h.

Following incubation in the MicroFoss instrument, the vials were removed and the contents of all vials, both presumptive positive and negative vials, were streaked out onto Polymixin Acriflavine Lithium Chloride Ceftazidime Esculin Mannitol (PALCAM) agar. Plates displaying small (2 mm diameter) gray-green colonies with a black sunken centre and black halo against a cherry-red background underwent further confirmatory testing for the presence of *Listeria* spp.

2.4. Listeria standard culture method

The standard culture method for *Listeria* spp. was performed as described in the Health Canada, Health Protection Branch Compendium of Analytical Methods (MFHPB-30, "Isolation of *Listeria monocytogenes* from all Food and Environmental Samples") (Pagotto et al., 2001). Ninety milliliters of UVM Modified Listeria Enrichment Broth (LEB) was added to each stomacher bag containing 10 ml of sample suspension. All stomacher bags were then stomached for 2 min and incubated at 30 °C for 24 and 48 h. At both 24 and 48 h, 0.1 ml of each incubated broth was aseptically transferred to tubes containing 9 ml of Modified Fraser's broth (MFB) as well as 0.1 ml of MFB supplement. Following incubation, all MFB tubes displaying a darkened broth (e.g. black, dark brown or dark green) were streaked onto both Oxford agar (OXA) and Listeria Plating medium (LPM) agar. OXA plates were incubated at 35 °C for 48 h, while LPM plates were incubated at 30 °C for 48 h. Both sets of plates were examined at 24 and 48 h of incubation. Suspect colonies on OXA were small (1 mm diameter) and black and displayed surrounding black haloes after 24 h. At 48 h, suspect colonies were large (2–3 mm diameter), black colonies displaying black haloes as well as a sunken centre. On LPM, suspect colonies appear white, with a blue-gray sheen developing as growth continues. Suspect colonies on both OXA and LPM were used to carry out confirmation testing.

2.5. Confirmation of Listeria suspect colonies

The first step in the test procedure for confirmation of *Listeria* spp. suspect colonies involved stab inoculation of a Trypticase Soy agar (TSA) plate, containing 5% horse blood, and subsequently streaking out the same colonies for purification onto a non-selective medium (e.g. TSA). Colonies displaying β-hemolysis ($\geq 1-2$ mm clearing) as well as those lacking hemolytic properties were tested further.

The pure cultures were used to inoculate sets of sugars consisting of mannitol, rhamnose and xylose. Acid production resulting in a colour change from purple to yellow indicated a positive result with respect to a microorganisms' ability to utilize a particular sugar. Catalase activity tests (positive if gas bubbles were present), motility tests (short rods with slight rotating or tumbling motion) and Gram staining (Gram-positive) were also included in the confirmation process. Results from all of the aforementioned tests were combined to confirm the presence of *Listeria* spp. in a particular sample. The

results were also used to speciate any *Listeria* spp. present.

2.6. MicroFoss false positive cultures

Microorganisms producing false positive esculin hydrolysis in the MicroFoss instrument were cultured and isolated. Pure cultures of the isolates were identified using a fatty acid-based bacterial identification system developed by Microbial ID (MIDI), Newark, DE. Each culture was passaged twice at temperatures of 28 °C (on Trypticase Soy Broth Agar—TSBA) as well as 35 °C (on Blood Agar—BA), harvested after 24–72 h of growth (35–45 mg, wet weight) and stored at −40 °C. Fatty acid extraction and analysis was performed as described in the MIDI system operating manual (Microbial ID, 1993). Pure cultures of isolates underwent four critical steps: saponification, methylation, extraction and washing. Each extracted organic layer was then injected into the MIDI gas chromatograph system and fatty acid methyl ester (FAME) patterns were produced. Analysis of the FAME patterns and comparisons with a library database of microbial FAME patterns allowed for microbial identification to the species level.

2.7. Estimation of sensitivity and specificity

The sensitivity and specificity of each method was determined using the following equations (de Boer and Beumer, 1999);

$$\text{Sensitivity } (\%) = \frac{\text{number of true positives } (p)}{p + \text{number of false negatives}} \times 100$$

$$\text{Specificity } (\%) = \frac{\text{number of true negatives } (n)}{n + \text{number of false positives}} \times 100$$

The sensitivity value determined indicates the capability of a given detection method to detect the presence of a specified microorganism, while the specificity value defines the ability of a given detection method to differentiate between the specified microorganism and interfering microorganisms.

3. Results

The numbers of false positive, true positive, false negative and true negative results are summarized for the 552 environmental samples tested (Table 1). The sensitivities of the MicroFoss and standard culture methods are similar. The MicroFoss method detected *Listeria* spp. in 114 samples whereby the standard method detected 117 positive samples. Twelve of the positive samples were detected by the MicroFoss method and not by standard culture and 15 samples were detected by the standard culture method but not by the MicroFoss method. This result may be due to the fact that the environmental sponge samples contained low levels of *Listeria* spp., and this may have resulted in an uneven distribution of the *Listeria* spp. present in the split samples tested. Clearly, both the MicroFoss and standard culture methods show analogous sensitivity values of 88.4% and 90.7%, respectively, when the total number of positive results obtained by both methods are used for determining sensitivity. The test results from experiments using spiked samples demonstrated that the detection limit

Table 1
Sensitivity and specificity of the MicroFoss system in relation to the standard culture method for the detection of *Listeria* species in 552 environmental samples

	MicroFoss method		Standard culture method	
	Number of samples	Percentage of total samples (%)	Number of samples	Percentage of total samples (%)
False positive	31	5.6	N/A[a]	N/A
True positive	114[b]	20.7	117	21.2
False negative	15	2.7	12	2.2
True negative	392	71.0	423	76.6
Sensitivity (%)	97.4 (88.4)		100 (90.7)	
Specificity (%)	92.7		100	

() Percent sensitivity relative to the total number positive tests obtained by both methods.

[a] Not applicable.

[b] Result includes MicroFoss *Listeria* true positive tests that are negative by reference culture method.

of *L. monocytogenes* (1 ATCC strain and 5 lab strains), *L. innocua* and *L. welshmeri* were also detected at 1 cfu/ml while the *L. ivanovii* strain tested was detected at 100 cfu/ml.

The specificity of detection of *Listeria* spp. by the MicroFoss method was 92.7% in comparison to standard culture (Table 1). The false positives (5.6%) of the naturally contaminated environmental samples were readily confirmed by culture and Gram stain of presumptive positive vials. When samples were allowed to incubate in the MicroFoss instrument for an additional 18 h after the initial 30 h of incubation, 61 samples showed positive results. However, all of the additional positive samples obtained after the 30 h of incubation were false positives.

Six *Listeria* spp. were detected and cultured by the MicroFoss and standard methods (Table 2). Both methods recovered *L. monocytogenes* more frequently than other *Listeria* species (89.4%—MicroFoss, 88.0%—Culture). *L. innocua* was cultured second-most frequently, with similar rates for both methods (8.8%—MicroFoss, 7.7%—Culture). *Listeria seeligeri* was detected by both the MicroFoss (0.9%) and Culture (2.5%) methods. The MicroFoss method also detected *L. ivanovii* in one sample (0.9%). The standard method was able to detect species of *Listeria grayi* and *L. welshmeri* at the same frequency (0.9%). Notably, the MicroFoss system was shown to detect *L. welshmeri* in spiked samples.

In order to determine the type of microorganisms producing false positive results in the MicroFoss system, positive vials were cultured on PALCAM agar. The results are summarized in Table 3. The group of microbes causing the most frequent number of false detections (48.4%) displayed medium, yellow-gray, filamentous morphology and were long motile rods (few in chains) that stained Gram-positive. Based on fatty acid composition, one culture from this group was identified as *Bacillus pumilus*. Microbes displaying pin-point pale morphology, and were short motile rods that stained Gram-positive were second (35.5%) in terms of their frequency in causing false detection results. Fatty acid extraction and identification testing determined the presence of microbes such as *Clavibacter michiganense* and *Corynebacterium aquaticum*. Colonies that demonstrated bacilli-like morphologies were motile rods as well as Gram-positive. The bacilli-like microbes were found to cause false detection at a lower frequency

Table 3
Summary of groups of microorganisms cultured from 31 environmental samples that were falsely detected by the MicroFoss instrument

Microbial identification	Microbe description	No. of false positive Samples	Percentage of total false positive samples (%)
Bacillus pumilus	Yellow-gray, filamentous, Gram-positive, long motile rods	15	48.4
Clavibacter michiganense, *Corynebacterium aquaticum*	Pin-point, pale, Gram-positive, short motile rods	11	35.5
Bacillus pumilus	Bacilli-like, Gram-positive, motile rods	5	16.1

Table 2
Summary of *Listeria* species detected from the environmental samples that tested positive by the MicroFoss and/or standard methods

	MicroFoss method (114 positive samples)		Standard culture method (117 positive samples)	
	Number of positive samples	Percentage of total positive samples (%)	Number of positive samples	Percentage of total positive samples (%)
L. monocytogenes	102	89.4	103	88.0
L. innocua	10	8.8	9	7.7
L. seeligeri	1	0.9	3	2.5
L. grayi	ND[a]	–	1	0.9
L. welshmeri	ND	–	1	0.9
L. ivanovii	1	0.9	ND	–

[a] Not detected.

(16.1%) vs. the two previously mentioned microorganisms. *B. pumilus* was identified from the bacilli-like group using fatty acid extraction. Clearly, *Bacillus* spp. were the most frequent cause of false positive test results for the MicroFoss system; however, they are easily distinguishable from cultures of *Listeria* spp.

4. Discussion

In this study, the MicroFoss and standard culture methods for *Listeria* spp. had similar sensitivities of detection of *Listeria* spp. in environmental sponges. Also, the MicroFoss and standard methods had similar numbers of false negative results. These false negative results may have been due to the limitation of the sample preparation procedure involving the use of split samples from each sponge swab. Split samples were used in order to avoid uneven distribution of *Listeria* organisms on the swabs, which were to be tested by both methods. However, at very low levels of *Listeria* (e.g. 1 cfu/sponge) it is possible that one of the split samples contained *Listeria* while the other did not. Although the sample preparation procedure used in this study can be considered a limitation, this is the only way a single sponge sample can be used to assess the performance of both methods. The use of duplicate samples would have introduced an additional variable in the test results.

The false positive results achieved by the MicroFoss system may be due to a number of different factors. Firstly, heavily soiled samples (e.g. caked dirt) may have harboured a large number of microorganisms other than *Listeria* spp. Excessive microbial activity may have overwhelmed the inhibitory system (e.g. selective supplement) such that certain microbes (mainly Gram-positive bacilli) were able to utilize some of the nutrients available in the medium possibly resulting in esculin hydrolysis and thus false detection. All presumptive positive results obtained by the MicroFoss system after 30 h of incubation time were false positives. Therefore, incubation of samples beyond 30 h is not recommended.

As with any inhibitory system, the majority of competitive microbes are inhibited; however, there are always a select few microbes that are capable of coping with inhibitory situations at specific microbial concentrations. An example of one such microbe is a strain of *B. cereus* (lab strain B52, BioSys lab) that is capable of overcoming the *Listeria* inhibitory system when present at concentrations of $\geq 10^3$ cfu/g. Therefore, false detection results may have also been the result of groups of microbes that were resistant to the established inhibitory system.

A study by El-Shenawy (1998) determined that the two most prevalent species of *Listeria* are *L. monocytogenes* and *L. innocua* in a dairy food-processing environment. The 1998 study revealed that *L. innocua* (32%) was more prevalent in the processing plant environment versus *L. monocytogenes* (13%). Our study also concluded that *L. monocytogenes* and *L. innocua* are the two most common species of *Listeria* in the food processing plant environment sampled in this study. However, in our study the prevalence of *L. monocytogenes* was higher than that of *L. innocua*. This variation may be due to a few factors, namely the difference in type of food processing plant (dairy vs. vegetable) as well as the difference between sampling methods (non-biased vs. biased).

A study by Firstenberg-Eden and Shelef (2000) involving the testing of spiked raw and processed food samples for *Listeria* spp. using the MicroFoss method did not report any false negative results and the false positive rate was somewhat higher (11.6%). The Firstenberg-Eden and Shelef study did not compare the MicroFoss method against a standard culture method for *Listeria* and therefore sample splitting was unnecessary. The lack of sample splitting may have contributed to the lack of false negative results. The study determined that the majority of microorganisms causing false positive results were Gram-positive cocci (e.g. *Staphylococcus aureus*, *Enterococcus faecalis*); however, four false positive results were the result of bacilli growth. In contrast, the majority of microorganisms causing false positive results in the present study were Gram-positive bacilli.

The MicroFoss and standard culture methods for *Listeria* can be compared on a few different aspects. In terms of time length of the procedure, a presumptive positive sample can be detected within 30 h with confirmed positive cultures obtained within 72 h using the MicroFoss method, while the standard culture method required a minimum of 6 days. With

respect to labour, the MicroFoss method required less hands-on time, in that there were fewer transfers and less plating, in comparison to the standard culture method. Overall, the MicroFoss system is considerably more efficient in terms of time and labour.

There are a variety of rapid *Listeria* test systems available for use by food production facilities. For example, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) technologies may also be used for screening purposes; however, these methods require a higher level of expertise and requirements for pure cultures or high numbers of *Listeria* organisms in culture (Peng and Shelef, 2000; Lawrence and Gilmour, 1994). Other rapid test kits are available and depend on the presence of high numbers of *Listeria* organisms in culture, and are not automated (Warburton and Boville, 2001). Notably, 128 samples or more can be tested using the MicroFoss system, thereby providing a cost efficient method for screening large numbers of environmental samples for *Listeria* spp.

5. Conclusions

This study has shown that the MicroFoss system is comparable to the standard culture method in terms of sensitivity. Although low numbers of false positive results can occur because of lack of inhibition of some competing microorganisms such as *Bacillus* species from environmental samples, all of the microorganisms causing false positive detection were easily distinguishable from *Listeria* spp. based on simple morphological features.

The simplicity, semi-automation and timeliness of the MicroFoss system are definite advantages, making it highly adaptable to in-plant use. The use of this system will allow food production facilities to determine which plant areas are prone to contamination by *Listeria* spp. This information can be used to improve plant sanitation practices and to develop preventative actions against contamination of any type of food product with *Listeria* spp.

References

Bell, C., Kyriakides, A., 1998. *Listeria*: A Practical Approach to the Organism and its Control in Foods Blackie Academic and Professional, London, UK.

BioSys, 2001. BioSys Operator Manual BioSys, Ann Arbor, MI.

Blackman, I., Frank, J., 1996. Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. J. Food Prot. 59, 827–831.

de Boer, E., Beumer, R., 1999. Methodology for detection and typing of foodborne microorganisms. Int. J. Food Microbiol. 50, 119–130.

El-Shenawy, M., 1998. Sources of *Listeria* spp. in domestic food processing environment. Int. J. Environ. Health Res. 8, 241–251.

Firstenberg-Eden, R., Shelef, L., 2000. A new rapid automated method for the detection of *Listeria* from environmental swabs and sponges. Int. J. Food Microbiol. 56, 231–237.

Lawrence, L., Gilmour, A., 1994. Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. Appl. Environ. Microbiol. 60, 4600–4604.

Microbial ID, 1993. Microbial Identification System Operating Manual, Version 4 Microbial ID, Newark, DE.

Murray, P., Baron, E., Pfaller, M., Tenover, F., Yolken, R., 1995. Manual of Clinical Microbiology, 6th edition ASM Press, Washington, DC.

Pagotto, F., Daley, E., Farber, J., Warburton, D., 2001. Isolation of *Listeria monocytogenes* from all food and environmental samples. In: Health Canada—Compendium of Analytical Methods, MFHPB-30. Polyscience Publications, Laval, Quebec, pp. 1–15.

Peng, H., Shelef, L., 2000. Rapid detection of low levels of *Listeria* in foods and next-day confirmation of *L. monocytogenes*. J. Microbiol. Methods 41, 113–120.

Peng, H., Shelef, L., 2001. Automated simultaneous detection of low levels of listeriae and salmonellae in foods. Int. J. Food Microbiol. 63, 225–233.

Warburton, D., Boville, A., 2001. Detection of *Listeria* spp. in foods using the Oxoid Listeria rapid test. In: Health Canada—Compendium of Analytical Methods. Polyscience Publications, Laval, Quebec.

Wong, A., 1998. Biofilms in food processing environments. J. Dairy Sci. 81, 2765–2770.