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Comparison of Media and Methods for Detecting and Enumerating *Listeria monocytogenes* in Refrigerated Cabbage

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Direct plating, selective enrichment, and cold enrichment followed by secondary selective enrichment procedures were compared for detecting and enumerating *Listeria monocytogenes* in chopped cabbage stored at 5°C for up to 64 days. Addition of Fe^{3+} to solid media enhanced detection of the organism. Cold enrichment (5°C) in nutrient broth and brain heart infusion broth followed by secondary enrichment (48 h, 30°C) in Trypticase soy-yeast extract-antibiotic broth and thiocyanate-nalidixic acid broth and plating on selective agar media (Doyle and Schoeni selective enrichment agar [minus acriflavin hydrochloride, supplemented with 5 μg of Fe^{3+}/ml] and McBride *Listeria* agar) resulted in the detection of highest populations.

Listeria monocytogenes is present on vegetation (6, 7, 9, 11), some of which is destined for human consumption. Vegetables which may be contaminated on the farm or during prolonged cold storage without subsequent cooking represent vehicles for transmission of listeriosis.

Contaminated foods that have caused listeriosis have been largely of animal origin. However, a large outbreak (41 cases) has been documented (9) in which cabbage was the incriminated source of *L. monocytogenes*. Experiments in our laboratory have shown that the microorganism can grow on refrigerated cabbage and in cabbage juice over a wide pH range and with up to 5% NaCl (1, 2).

To date, a comparison of methods for detecting and enumerating *L. monocytogenes* from vegetation has not been reported. Presented in this paper are the results of an investigation of selected media and methods for detecting and enumerating the organism in refrigerated chopped cabbage.

MATERIALS AND METHODS

Strain used and preparation of inoculum. The strain of *L. monocytogenes* used in all tests was LCDC 81-861 (serotype 4b), an isolate from raw cabbage. Stock cultures were maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) slants at 4°C. Tryptic phosphate broth (1) was used to culture the organism in preparation for inoculating cabbage; 48-h-old cultures incubated at 30°C were diluted in 0.1 M potassium phosphate buffer (pH 7.0) to give a viable cell population ranging from 1.0×10^5 to 1.8×10^5/ml.

Preparation of cabbage and inoculation procedure. Fresh cabbage was chopped in a Hobart Silent Chopper (model 84142; Hobart Manufacturing, Troy, Ohio) into pieces not exceeding 1 cm in length. For each experiment, chopped cabbage (1,500 g) was combined with 1,500 ml of the buffer suspension (22°C) of *L. monocytogenes*. After 5 min of intermittent mixing, the cabbage was drained and stored at 5°C in a covered stainless steel container. A separate uninoculated control lot of cabbage was stored at 5°C.

Media and procedures for detection and enumeration. Cabbage was thoroughly mixed immediately before samples were taken for analyses. Three separate experiments were conducted.

(i) Experiment 1. Duplicate cabbage samples (100 g each) stored at 5°C for 0, 9, 25, 39, and 64 days were combined with 200 ml of buffer in a Colworth Stomacher bag and pummeled for 1 min. The wash fluid was serially diluted in buffer and surface plated (0.1 ml) in duplicate onto test media. The test media used were gum base-nalidixic acid medium (GBNA) (7), Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-antibiotic medium (TSYEA) (J. Lovett, D. W. Francis, and J. M. Hunt, J. Food Prot., in press), Doyle and Schoeni selective enrichment medium (DSSE) (4), DSSE minus acriflavine hydrochloride (DSSE − A), DSSE minus acriflavine hydrochloride and sheep blood (DSSE − AB), thiocyanate-nalidixic acid medium (TN) (10), TN plus 0.2% glucose (TN + G), tryptose blood agar plus 1.0% glucose (TBG) (11), McBride *Listeria* agar (MLA) (8), and MLA minus sheep blood (MLA − B). These media supplemented with ferric citrate (+ Fe) to give a concentration of 5 μg of Fe^{3+} per ml were also evaluated in experiment 1. All media except GBNA contained 1.5% agar. Colonies were observed for color, shape, and size and were counted after 48 h of incubation at 30°C; microscopic examination and Gram reaction were carried out on cells from suspect colonies.

Plate count agar (PCA; Difco) and MRS agar (Oxoid Ltd., London, England) supplemented with 0.1% sorbic acid (MRSS, pH 5.4) after heat sterilization were included in the battery of test media to determine aerobic and lactic acid bacterial populations, respectively. Aerobic and lactic acid bacterial populations were enumerated after 48 h at 30°C.

(ii) Experiment 2. Cabbage stored at 5°C for 0, 14, and 30 days was subsampled and treated as described in experiment 1 in preparation for immediate surface plating (0.1 ml) on DSSE − A supplemented with 5 μg of Fe^{3+} per ml (DSSE − A + Fe), MLA, and PCA. Iron was included in the media, since Fe^{3+} has been reported to enhance the growth rate of virulent strains of *L. monocytogenes* (3). Colonies were observed and enumerated after 48 h at 30°C.

In a separate test, 20-g subsamples of cabbage stored for 0, 14, and 20 days were combined with 100 ml of each of five selective media (TSYEA, DSSE, DSSE − A, TN, and TN + G) not containing agar, as well as Stuart transport medium

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(ST; Difco) and brain heart infusion broth (BHI; Difco), in 250-ml Erlenmeyer flasks. Likewise, 20-g subsamples were also combined with 100 ml of each of these seven broths supplemented with 5 μg of Fe^{3+} per ml. After 48 h at 30°C, enrichment samples were vigorously shaken, and the broth was serially diluted and plated on DSSE − A + Fe, MLA, and PCA. Colonies were observed and enumerated after 48 h at 30°C.

(iii) Experiment 3. Fresh inoculated and uninoculated cabbage was subsampled (20 g) and combined with 100 ml of nutrient broth no. 2 (Oxoid) and BHI broth in 250-ml Erlenmeyer flasks. After 0, 14, and 30 days at 5°C, cold-enriched mixtures were vigorously shaken, and 0.1 ml of serially diluted broth was surface plated (0.1 ml) on DSSE − A + Fe, MLA, and PCA. Colonies were observed and enumerated after 48 h at 30°C.

In a separate test, culture broth (0.5 ml) from cold-enriched samples was also inoculated into TSYEA, DSSE − A + Fe, TN not containing agar, and BHI and held at 30°C for 48 h before 0.1 ml of serially diluted samples was surface plated on DSSE − A + FE, MLA, and PCA. Colonies were counted after 48 h at 30°C.

Diluted cell suspensions of a pure culture of *L. monocytogenes* were also subjected to the same scheme of cold-enrichment procedures.

RESULTS AND DISCUSSION

Results from experiment 1 indicated that GBNA, DSSE, DSSE + Fe, DSSE − A + Fe, TN + G + Fe, and MLA performed better than other test media. Superior performance was judged by the ability of media to support colony formation and the ease of recognition of *L. monocytogenes* colonies with the naked eye. Since the total aerobic population increased from 3×10^5/g at the initiation of the experiment to 2×10^9/g after 39 days of incubation, DSSE − A + Fe and MLA emerged as the most satisfactory media for enumeration of *L. monocytogenes*. The initial population of *L. monocytogenes* in cabbage was 2×10^4/g; the population increased to 5×10^6/g after 9 days and then gradually to 8×10^7/g on DSSE − A + Fe and MLA during the remainder of the 64-day test period. Populations ranging from 3×10^4 to 1×10^7/g were detected on the other test media. Colonies formed on DSSE − A + Fe were 1 to 2 mm in diameter, smooth, light yellowish-brown, and with a raised dark reddish-brown center on top and a dark reddish-brown center on the reverse side. The presence of Fe^{3+} in the medium greatly facilitated recognition of *L. monocytogenes* in that the center of each colony was dark reddish-brown, especially on the reverse side.

Colonies of *L. monocytogenes* formed on MLA were 1 to 2 mm in diameter with a dense, nearly white center changing to slightly bluish-grey toward the circumference. Colonies were relatively easy to distinguish from those formed by other microflora in the cabbage.

While most other test media supported good growth of pure cultures of *L. monocytogenes*, the colonies formed by the microorganism were often visually indistinguishable from colonies formed by other bacteria present on the cabbage. This problem was magnified after 39 and 64 days of storage, when the total aerobic plate count had increased to $>10^9$/g. The Henry method of oblique lighting (5) was not particularly useful in detecting *L. monocytogenes* colonies. Other bacteria present in uninoculated cabbage also gave a characteristic blue color when examined by this procedure.

The lactic acid bacterial population reached 2.6×10^8 CFU/g on day 25 of storage and then declined substantially at 39 and 64 days. The presence of these or other types of bacteria in cabbage did not appear to inhibit the proliferation of *L. monocytogenes* during the 64-day storage period. The microorganism is not particularly sensitive to low pH in stored cabbage (2).

Results from experiment 1 led to the conclusion that direct plating of cabbage samples onto solid selective and semiselective media is not an acceptable procedure for the detection and enumeration of *L. monocytogenes*. Experiment 2 was therefore conducted to test the suitability of selective enrichment broths for enhancing the growth and detection of *L. monocytogenes* in cabbage. Direct plating on DSSE − A + Fe, MLA, and PCA was also done to compare the methods.

Initial analyses (day zero) revealed that eight selective enrichment broths (TSYEA, TSYEA + FE, DSSE, DSSE + Fe, TN, TN + Fe, BHI and BHI + Fe) supported the development of the largest populations (6×10^8 to 1×10^9/ml) of *L. monocytogenes* during the 48-h enrichment period at 30°C. DSSE − A + Fe and MLA performed equally well as solid enumeration media following enrichment; 9×10^8 to 1×10^9/g of cabbage were detected. TSYEA and TN were clearly the best broths for selectively enriching for *L. monocytogenes* after the cabbage had been stored at 5°C for 14 and 30 days.

Experiment 3 was designed to determine whether any benefit might be derived from cold enrichment (14 and 30 days) in nonselective broths (nutrient broth no. 2 and BHI) before either direct plating (DSSE − A + Fe and MLA) or subjecting to a secondary enrichment (TSYEA and TN broths, 48 h, 30°C) before direct plating on DSSE − A + Fe and MLA). Secondary enrichment after cold enrichment for 14 days enhanced the recovery of *L. monocytogenes* as judged by the largest number of colonies (9×10^6/g) detected on DSSE − A + Fe and MLA; 2×10^7/g were detected after 30 days. This procedure was deemed the most satisfactory for detecting *L. monocytogenes* in refrigerated cabbage. Evaluation of the procedure by using a wide variety of vegetation as test material is necessary before methods for detecting and enumerating the organism on fresh produce can be recommended. Methods for detecting low populations (e.g., <10/100 g) must be developed on the basis of results of these experiments.

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