Confirmation and identification of *Listeria* species from fresh lettuce

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Abstract

The food borne pathogen bacteria included in genus Listeria are the causative agents of listeriosis, a severe disease with high hospitalization and case fatality rates. Listeria monocytogenes can survive and grow over a wide range of environmental conditions such as refrigeration temperatures, low pH and high salt concentration. Foods identified as high risk include refrigerated, minimally processed products such as fresh-cut salads and also in fresh fruits and vegetables.

The incidence of Listeria species in the fresh lettuce from traditional market and supermarkets in Romania was studied during the period of January 2008–July 2008. Lettuce were sampled and tested for presence of Listeria spp. by using two step enrichment procedure, followed by plating on two selective agars Palcam and Oxford. The confirmation of the isolates was based on biochemical identification.

The contamination of Listeria monocytogenes found in Romanian fresh lettuce samples was identified on the lettuce grown in glass houses where the soil was fertilized with sheep feces and also in the sample from Supermarket C.

Keywords: Lettuce, incidence food borne pathogens, biochemical identification, *Listeria spp*., *Listeria monocytogenes*

Introduction

Fresh fruit and vegetables are essential components of the human diet and there is considerable evidence in favour of the health and nutritional benefits associated with the consumption of fresh vegetables.

When vegetables are consumed raw, as is the case with salads, harmful microorganisms may be present and ingested. Traditionally, eating raw fresh vegetables from the field was considered safe; however, bacterial pathogens are currently being found in or on fruits and vegetables [1,2].

To minimize the risk of infection or intoxication associated with the consumption of raw vegetables, potential sources of contamination from the environment to the table should be identified and specific measures and interventions to prevent and/or minimize the risk of contamination should be considered and correctly implemented [2]. Surveys of minimally-processed fruit and vegetables and sprouts have demonstrated that fresh-cut produce could harbour high counts of bacteria and also food borne pathogens such as *Salmonella*, *Listeria monocytogenes*, *Aeromonas hydrophila* and *E. coli O157:H7* [2,3,4,5].

The *Listeria monocytogenes* bacterium is of particular importance as it can cause humans listeriosis; this infection is responsible for an estimated 2500 serious illnesses and 500 deaths each year in the United States. During the last decade *Listeria monocytogenes* has been recognized as an agent of food borne illness. Healthy people rarely contract listeriosis, but the illness can be serious for some people, especially the elderly, newborns, pregnant women, and those with weakened immune systems. Also, unlike most food borne pathogens, *Listeria monocytogenes* multiplies readily in refrigerated foods that have been contaminated.

Several sanitizers have been investigated for use in controlling bacteria in fresh produce; for example, chlorinated water, chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), organic acid, calcinated calcium solution, and ozonated water have been evaluated against pathogens on produce [6]. Acidic electrolyzed water (AcEW), which is produced by the electrolysis of an aqueous sodium chloride solution in an anode cell, has also been reported to have a strong bactericidal effect on most pathogenic bacteria in lettuce [7], alfalfa seeds, sprouts [8] and tomato [9].
The aim of the study was to highlight the pathogen bacteria of *Listeria* genus from lettuce which's traded on different places, supermarket and traditional market in Romania.

Materials and Methods

Materials

A number of 4 samples of lettuce from different sources were analyzed during the period January 2008–July 2008. First sample was from a glass house where a traditional cultivation method was used with sheep feces as fertilizer. The second sample was from "A" supermarket (originated from Romania); the third sample was from "B" supermarket (originated from Romania) and the fourth one was from "C" supermarket (originated from Germany). Only the last sample was packed.
Bacterial growth mediums: for the primary growth (Semi Fraser broth), the secondary growth (Fraser broth), isolation (Oxford Agar, Palcam Agar), were purchased from Merck, Germany. All others chemicals were at analytical grade.

Methods

Strains isolation

The *Listeria monocytogenes* detection and enumeration followed methods provided by the International Organization for Standards (ISO 11290-1:1997/Amd.1:2004) (Fig.1).

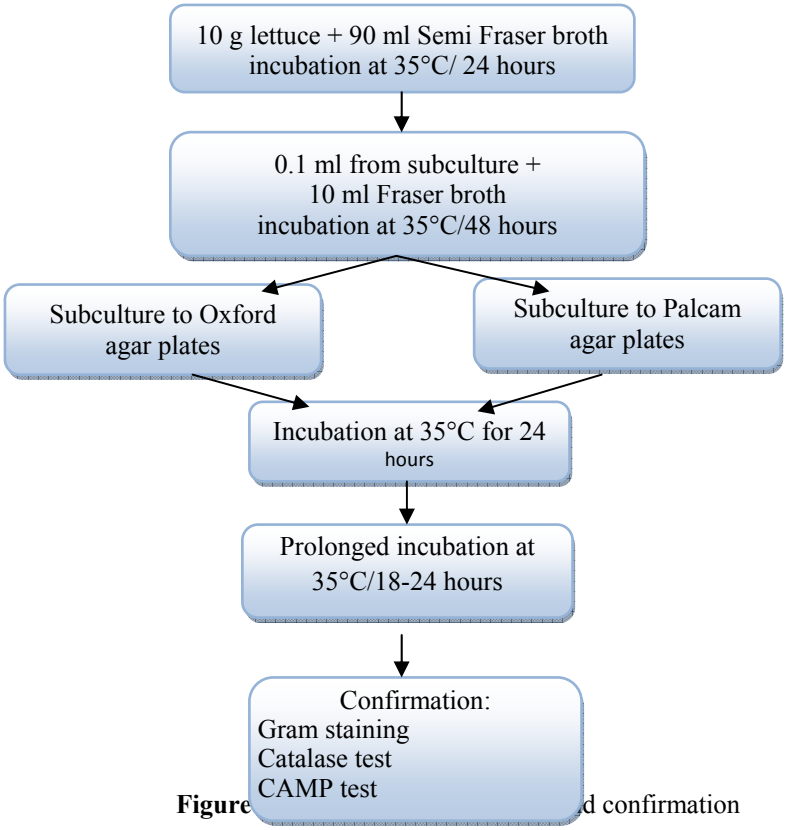

Figure d confirmation

Ten grams of a sample was aseptically taken, blended for 2 min in 90 ml Semi Fraser broth and incubated at 35 °C for 24 h. A volume of 0.1 ml of primary enrichments were transferred to 10 ml of Frazer broth and incubated at 35 °C for 48 h. Secondly enrichments were streaked on Oxford agar and Palcam agar and incubated at 35 °C for 24 h. The plates were examined for typical *Listeria* colonies (black colonies with black sunken) and if no colonies were obtained a supplementary incubation until 24 h was done.

Confirmation and identification steps

All the isolates were subjected to standard identification tests such as Gram staining and catalase test. For further confirmations of *Listeria spp*., CAMP test was performed according to the Bergey's Manual of Systematic Bacteriology [10].

Gram staining (or Gram's method) was used for differentiating bacterial species into two large groups, Gram-positive and Gram-negative bacteria, based on the chemical properties of their cell walls. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique, thus forming *Gram variable* and *Gram indeterminant* groups as well.

Catalase test. The presence of catalase enzyme in the test isolate is detected by using hydrogen peroxide. If the bacteria possess catalase (i.e. are catalase positive), when a small amount of bacterial isolate is added to hydrogen peroxide and bubbles of oxygen are observed.

CAMP test. For the CAMP test, fresh isolates of beta-hemolytic *Staphylococcus aureus* and *Rhodococcus equi* were streaked vertically on a sheep blood agar plate. The vertical streaks are separate so that test strains may be streaked horizontally between them without touching the vertical streaks. After 24-48 h incubation at 35°C, the plates for hemolysis in the zone of the vertical streaks were examined.

Results and Discussions

First step of strains isolation

On Fraser broth, colonies were 0.2 to 0.8 mm in diameter, smooth, punctiform, bluish gray, translucent, and slightly raised with a fine surface texture and entire margin after 24h of incubation. When cultures of *Listeria* grown for 18 to 24 h at 35^{o}C on a clear medium of Palcam were examined with a stereomicroscope under transmitted light, the smooth colonies exhibited a typical blue-green iridescence (Fig. 1).

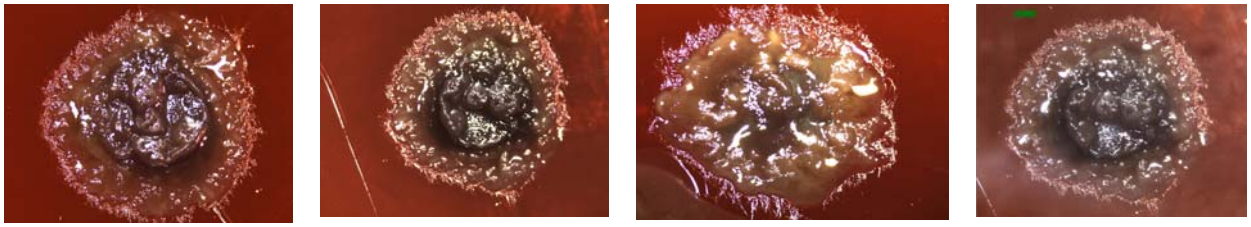

Figure 1. *Listeria* spp. colonies from samples (A-greenhouse, B-supermarket A, C- supermarket B, D-supermarket C) examined with a stereomicroscope

***Listeria* species confirmation and identification**

Gram staining. *Listeria* was identified as a small (0.5 µm in diameter and 1 to 2 µm in length), regular Gram-positive rod with rounded ends. Cells were found as single units or in short chains or they may be arranged in V and Y forms or in palisades (Fig. 2). Sometimes,

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cells were coccoid, averaging about 0.5 µm in diameter and may be confused with streptococci. In old cultures, some cells lose the ability to retain Gram stain and may be occasionally mistaken for *Hemophilus*.

In old and rough culture and after osmotic shock cells can appear as long, thin, filamentous. *Listeria* does not produce spores and capsules are not formed.

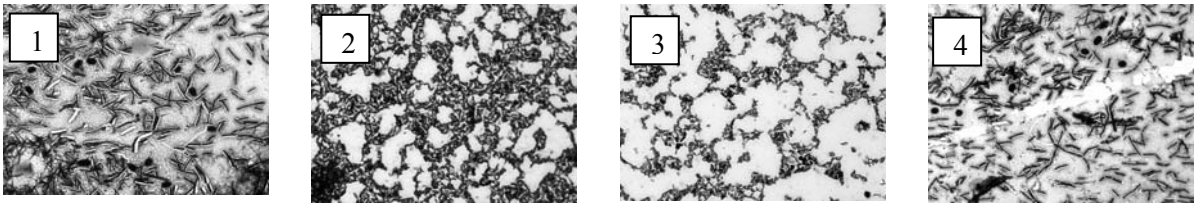

Figure 2. Gram staining evaluation of *Listeria* from samples
(1-greenhouse, 2-supermarket A, 3- supermarket B, 4-supermarket C)

Catalase test. During the catalase test it was observed the presence of gas bubbles in all studied samples. When hydrogen peroxide was added on the suspension which was obtained from Fraser broth colonies it was observed the gas bubbles deliberation (Figure 3).

Figure 3. Catalase test for *Listeria* sp. presence confirmation

CAMP test indicated the increases of *Listeria monocytogenes* and *Listeria innocua* hemolysis close to the *Staphylococcus* streak, while *Listeria ivanovii* hemolysis is only observed close to the *Rhodococcus* streak. The test was able to differentiate *Listeria ivanovii* and *Listeria innocua*.

The CAMP test on the studied samples indicated the following results (Fig. 4):

- Glasshouse and Supermarket C samples: presence of *Listeria monocytogenes* that produce a light hemolysis;
- Supermarket A sample: presence of *Listeria ivanovii* that produce a strongly hemolysis;
- Supermarket B sample: presence of *Listeria innocua* that produce a medium hemolysis;

Figure 4. CAMP test evaluation of Listeria species

Conclusions

Listeria monocytogenes was identified on the lettuce grown in glass houses where the soil is fertilized with ship feces and also in the sample from supermarket C. This study demonstrates that the control of *Listeria monocytogenes* in food manufacturing and sale is

essential in order to minimize the potential for these bacteria to be present in fresh lettuce at the point of consumption at levels hazardous to health.

Listeria monocytogenes in food cannot be seen, tasted, or smelled. Common sense and simple precautions that apply to any food borne illness should be used.

Good sanitation, personal hygiene, and safe buying, storing, cooking and serving methods, when applied in home, retail and food service environments, can reduce the risk of problems with *Listeria monocytogenes.*

Although most of the population is at very low risk for listeriosis, the risk can be reduced if the consumers will cook all food of animal origin; will wash raw vegetables thoroughly before eating, will keep uncooked meats separate from vegetables, cooked foods, and ready-to-eat foods, will avoid to consume raw/unpasteurized milk or foods made from raw milk and will wash hands, knives, and cutting boards after handling uncooked foods.

Acknowledgements

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