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Listeria Survival and Growth in Newly Cutted Vegetables

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ABSTRACT

This study investigates the prevalence of *Listeria spp* in newly cutted vegetables. Theinfluence of initial head-spaces of air – 4.9% CO2, 2.1% O2, 93% N2 and 5% CO2, 5.2% O2, 89.8% N2 – on *Listeria monocytogenes* and on microbial association with shredded carrots and lettuce was studied at 4ºC. These pathogens survived but did not grow in any vegetable regardless of the packaging system used. Total viable count and lactic acid bacteria and *Pseudomonas* were also monitored. Lactic acid bacteria were the predominant organismsin all samples. The PH dropped significantly during the storage of vegetables. The selective medium mainly used for the isolation of *Listeria* is oxford agar. Using isolated *L.monocytogenes* from vegetables, morphologic and biochemical identification was carried out. The results conferred during this study indicate the contamination of *Listeria* in vegetables.

KEYWORDS: *L.monocytogenes, Lettuce, Listerosis*

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INTRODUCTION

Listeria monocytogenes is a gram positive, facultative anaerobic, opportunistic bacterial pathogen. It is the causative agent of listeriosis, a disease which predominantly affects the elderly, immunocompromised patients are affected and women who are pregnant, as well as their unborn or newborn children. Contaminated food stuffs and vegetables are the main cause of infection **(Garner and Kathariou 2016).** This microorganisms have the ability to withstand extreme in PH and low temperature. Although dairy products have gotten the most press as a cause of listeriosis, the bacterium has also been detected in beef, pig, chicken and sea food. Its presence on fruits and vegetables are salad vegetables such as cabbage, celery, lettuce, cucumber, onion, leeks, water cress and fennel found to contain high population of *L.monocytogenes.*

It is a harmful bacteria that can be found in refrigerated, ready-to-eat foods such as meat, dairy-unpasteurized milk and milk products and products harvested from soil contaminated with *L.monocytogenes* **(Cartwright *et al.*,2013).** It have been found in a wide range of fresh product samples **(Zhu *et al.*,2017)** and other minimally processed foods, according to many investigations.

Aside from the potentially tragic loss of life, *Listeria* outbreaks have substantial economic effects due to a loss of consumer trust and subsequent decline in product sales and related value **(Mc Cullum *et al.*,2013).** In the US, the bacterium has been found on individual fresh products such as cabbage. Potatoes, cucumber and raddish as well as other vegetables **(Heisick*et al.*,1989).**

When raw vegetables come into contact with dirt or animal manure used as fertilizer, they might become infected with *Listeria*. Listeriosis symptoms are similar to flu symptoms. Fever, nausea, diarrhoea, pains and nervous system imbalance are some of the symptoms. Without taking adequate care of one's body, one's chances of living a long and healthy life are slime to none. When you first notice signs of listeriosis or any other illness caused by a parasite,you should seek medical attention immediately. Without treatment, a food borne infection could kill those with weak immune system and even still birth of an unborn child. Although the frequency of listeriosis is minimal

when compared to other food borne bacteria, the disease's prognosis is frequently more severe.

It is a significant pathogen for many people, hence it's a priority pathogen for them and many countries. *Listeria* can also grow at refrigeration temperature **(Chan and Wiedmann 2009).**

As a result it possess a greater risk to consumer over other food pathogens such as *Salmonella* and *E.coli.* Fresh-cutted vegetables has a higher growth potential for food borne pathogens than produce with the peel or rind intact, because on the sliced surface, there are more nutrients available and refrigeration is the only method of preservation used **(Francis *et al*.,1999).** *Listeria monocytogenes* is a human pathogen that can be found in food, has been linked to a number of serious food borne illness. The bacterium is a psychrotroph. It can growand thrive in temperature as low as 8ºc **(Conway *et al*., 2000).** As a result chilled foods have become an issue. The serotypes 1/2a, 1/2b and 4b of *L.monocytogenes* are a particular concern,as they account for upto 96 percent of human listeriosis cases throughout the world **(Thompkin*et al*.,2002).**

Because of the high fatality rate associated with *L.monocytogenes* infection, the united states of food and drug administration and U.S department of agricultural food safety inspection service have established a zero-tolerance for *L.monocytogenes* ready-to-eat food and also freshly cut fruits and vegetables **(Thomas *et al.*, 2000).**

The fresh-cut produce processing and packaging is a fast growing industries that provides consumer with both convenient and nutritious food. In addition to the new problem may arise by the growth of this industry. There is a scarity of information on this subject of the microbial contamination of fresh-cut fruits and vegetables (**Bean *et al*., 1973).** As a result of the chemical and physical barriers, given by the peel or rind, which acts as a preservative, it prevents the growth of microorganisms on the surface **(Morbid *et al*.,1983).**

The impediment is removed during the fruit's preparation for the market for which couldlead to the creation of large food-producing population of food borne human pathogens, resulting in higher risk to one's health consequently. It's vital to find out what happened to sthem as well as the control of food borne infection on vegetables that has been freshly sliced. According to FDA, about 2500 people suffer from listeriosis in the USA annually. The mortality rate could be 20-30% of those who contract listeriosis. Many recent reports show contamination and prevalence of *Listeria* in freshly cut vegetables such as cabbage, corn, carrot, parsley.

Infections with *L.monocytogenes* have been reported in various parts of the world in association with fresh cutted vegetables **(Meldrum *et al*.,2009).** For ex: *L.monocytogenes* was responsible for death of ten people by food poisoning listeriosis outbreak in chopped celery in Texas in 2010 **(Gaul *et al*.,2013).** In 2011, 30 people were infected in Colorado, listeria was spread through infected melons and in California, a listeria outbreak associated with caramel apple contamination was recorded in 2014 **(CDC.2015).**

This tendency has persisted and preventing *Listeria* contamination in fresh fruits and vegetables, as well as listeriosis outbreak linked to fresh produce, is now a food safety issue. There are two types of disease syndromes caused by *L.monocytogenes.* Listeriosis is a bacterial infection caused by the invasive pathogen *Listeria monocytogenes.* That is, the organisms infects sterile body organs such as the liver, spleen, cerebral spinal fluid and blood **(Wing *et al.*, 2002).**

Diarrhoea and fever are common in healthy adults. Fever, diarrhoea, abortion or stillbirth are the most common symptoms **(Khoury*et al.*, 2012).** It can cause sepsis, pneumonia and meningitis in newborns. *L.monocytogenes* also cause bacterial infection, that is a non-invasive disease that usually manifests as a febrile gastroenteritis or non-invasive gastroenteritis. Associated with outbreaks caused by tainted deli meat, chocolate milk and cheese, corn and smoked fish.

In recent years, the number of food borne outbreaks caused by contaminated fresh fruitsand vegetables has been on the rise. The majority of outbreaks have been documented in the US, Europe, Canada and a few other countries. Australia and New Zealand have a smaller proportion of the outbreak. The bacterium can proliferate in the gastrointestinal tract after ingesting contaminated food products, causing acute or persistent illness. The sickness does not have any symptoms. It can take a long time for food to be digested as well as the development of disease *L.monocytogenes* can survive and move around inside the body without causing any visible symptoms between cells. *L.monocytogenes* begin to kill or harm bodily cells, which maintain the body alive. Illness symptoms might appear suddenly or gradually depending on the severity of the particular pathogen, it can take weeks to identify the strain of *L.monocytogenes* and state of one's immune system at the time of infection **(CDC, 2014; Maxlen Clark, 2016).**

SCIENTIFIC CLASSIFICATION

Domain: Bacteria
Phylum: Bacillota
Class: Bacilli

Order: Bacillales
Family: Listeriaceae
Genus: *Listeria*
Species: *L.monocytogenes*

According to studies, *L.monocytogenes*can colonise upto 10% of human gastrointestinaltracts **(Ramsay *et al*., 2007).** Nonetheless, veterinarians are more likely to recognise clinical diseases caused by *L.monocytogenes,* particularly meningocephalitis in ruminants. Its ability to grow at temperature as low as 0ºC allows it to multiply at refrigeration temperatures, considerably improving its ability to elude detection in human meals. *L.monocytogenes* can move within eukaryotic cells by explosive polimerization of actin filaments at 30 degrees celcius (known as comet tails or actin rockets) **(Pizarro *et al*., 2019)**

Pregnant mothers are often advised not to eat soft cheese such as Brie, Camembert, fetaand queso blanco fresco, which may be contaminated with and allow of *L.monocytogenes*, due to its frequent pathogenicity, causing meningitis in newborns (acquired transvaginally) **(Farver*et al*.,1991).** In newborns, it is the third most prevalent cause of meningitis. Through the intake of contaminated foods such as unpasteurised dairy or raw foods, *Listeria monocytogenes* can infect the host's brain, spinal cord membranes and/ or blood stream **(CDCsources 2015).**

Listeriosis is a disease caused by an invasive infection with *Listeria monocytogenes*, When an infection isn't invasive, any illness that develops as a result of it is referred to as febril gastroenteritis. *Listeria* infection can cause sepsis, meningitis (or meningoencephalitis), encephalitis, corneal ulcers, pneumonia and intrauterine or cervical infections in pregnant women, which can lead to spontaneous abortion (second to third trimester) or stillbirth. Survivors of foetomaternal listeriosis may develop granulomatosis infantiseptica, which is characterized by pyogenic granulomas that cover the entire body and cause physical retardation. The start of the aforementioned illness is generally proceeded by influenza-like symptoms, such as a persistent fever.

D-galactose residues on the surface of *L.monocytogenes* cam bind to D-galactosereceptors on the host cell walls. M cells and peyer's patches of the intestinal mucosa are the most common host cells. *L.monocytogenes* can cross the intestinal membrane and into the bodyonce attached to these cells.

Listeria is classified as a gram-positive belonging to the Clostridium sub-branch becauseits genome has a low G+C concentration. Six different species can be found. They are *Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Listeria seeligeri, Listeria welshimeri andListeria grayi.*

Only two of the genus are generally considered to be pathogenic, *L.monocytogenes* in humans and *L.ivanovii* in other mammals. However, there have been some reports of *L.seeligeri* and *L.ivanovii* **(Rocourt*et al*., 1986, Cummins *et al*., 1994)** causing illness in humans.

Listeriosis is caused by a pathogenic infection caused by *L.monocytogenes* and it usuallyaffects people who are predisposed due to an underlying disease. Diseases that affescts the immune system, such as cancer or AIDS, as well as other people who are vulnerable, such as the elderly, pregnant women and other new born babies and foetuses are all examples of this

L.monocytogenes infection is pathogenic. The objective of the study was to determine the survival and growth of *Listeria monocytogenes* in newly cutted vegetables.

MATERIALS AND METHODOLOGY

SAMPLE COLLECTION

The samples are collected from four different market. The samples are lettuce, cabbage, cucumber and carrots. The purchased samples were added in to sterile poly ethylene bag and transported to the laboratory for isolation of bacteria. The microbial analysis was done within1-3 h of sample collection.

ISOLATION OF BACTERIA

The serial dilution agar plate technique was used for the isolation of bacteria from vegetables. Enrichment media fraser broth and oxford agar are used for the isolation of bacteria.Samples were rinsed in tap water and cut with a sterile knives. 25g of samples were taken aseptically and homogenised using sterile mortar and pestle later the sample was crushed and serially diluted and inoculated by streaking into a sterile stomacher bag containing enrichment media fraser broth and incubated at 37º C for 24 hours (Ding et al. 2013).

IDENTIFICATION OF BACTERIA ISOLATES

The isolated bacteria were identified on the basis of morphological, cultural and biochemical characteristics.

MICROSCOPIC AND MORPHOLOGICAL CHARACTERIZATION:GRAM STAINING:

A Clean grease free slide was taken and a smear of the bacterial culture was made on it

with a sterile loop. The smear was air- dried and then heat fixed. Then it was subjected to the following staining reagents:

- Flooded with crystal violet for 1 min. Followed by washing with running distilled water.
- Again flooded with Gram's Iodine for 1 min followed by washing with runningdistilled water
- Then the slide was flooded with Gram's Decolourizer for 30 seconds.
- After that the slide was counter stained with safranin for 30seconds, followed by washing with running distilled water.
- The slide was air dried and cell morphology was checked under microscope

BIOCHEMICAL CHARACTERIZATION OF *LISTERIA*:

INDOLE TEST

The Test organism is inoculated into tryptone broth, a rich source of the amino acid tryptophan. Indole positive bacteria produce tryptophanase (**Hadley *et al*., 1957**), an enzyme that cleaves tryptophan, producing indole and other products. When Kovac's reagent (p- dimethyl amino benzaldehyde) is added to a broth with indole in it, a dark pink color develops. The indole test must be read by 48 hours of incubation because the indole can be further degraded if prolonged incubation occurs. The acidic pH produced by *Escherichia coli* limits its growth. Indole is a nitrogen-containing compound formed from the degradation of the aminoacid tryptophan The indole test is important because only certain bacteria form indole. Indole can be easily detected with Kovac's . After the addition of the reagent and mixing the contents,the tube is allowed to stand. The alcohol layer gets separated from this aqueous layer and, upon standing, the reddening of the alcohol layer shows that Indole is present in the culture, Thus, the formation of the red layer at the top of the culture indicates the positive test.

PROCEDURE

- Tryptone broth was prepared and sterilized at 121°c for 15 minutes.
- Inoculated the medium with test organism by using suitable technique.
- Incubated at 37c for 24 hours.
- Growth of the organism was observed and added one ml of kovac's reagent.
- Colour changes were observed and recorded the reagent

METHYL RED TEST

- MR-VP broth was prepared, sterilized dispensed into sterlile test tube.
- Inoculate the tubes with the test organism and incubate at 37°C for 24 hours
- After incubation, add 5-6 drops of methyl red solution and shake.
- Allow to stand for few minutes and read result

VOGES PROSKAVER'S TEST

- MR-VP broth was prepared, sterilized dispensed into sterlile test tube.
- Inoculate the tubes with the test organism and incubate at 37°C for 24 hours.
- After incubation add 0.2 ml of VP reagent A and reagent B and shake.
- Allow to stand for few minutes and read result

UREASE TEST

- The Christensen's urea agar medium was prepared, sterilized and dispensed intoSterile test tube
- Slants were made and incubated with test organism.
- The test tube were incubated at 37°C for 24 hours

MOTILITY TEST

- A Drop of Microbial cell suspension is placed on to a Centre of coverslip with the Help of an inoculation loop.
- Vaseline is placed on the four comer of coverslip.
- A clean cavity slide is placed carefully over the drop of cover slip.
- The slide is inverted quickly.
- Then it was observed under low power objective

CITRATE UTILIZATION TEST:

The citrate test utilizes Simmon's citrate medium (Hadley et al., 1957 to determine if a bacterium can grow utilizing citrate as its sole carbon and energy source. Simmon's media contains bromthymol blue, a pH indicator with a range of 6.0 to 7.6. Bromthymol blue is yellowacidic pH's (around 6), and gradually changes to blue at more alkaline pH's (around 7.6). Uninoculated Simmon's citrate agar has a Ph of 6.9, so it is an intermediate green color. Growthbacteria in the media leads to development of a Prussian blue color (positive citrate)

PROCEDURE:

- Prepared simmon' s citrate medium and poured in test tubes.
- Sterilized the medium at 121°c for 15 minutes.
- A well-isolated colony was picked from the surface of a primary isolation medium and inoculated as a single streak on the slant surface of the citrate agar tubes.
- Incubated the medium 37°c for 24 hours.
- Observed colour change after incubation period.

OXIDASE TEST

The enzyme oxidase that forms the part of electron transport system is possess by some bacteria the enzyme oxides the report the reagent N, N tetramethylene para phenylene diamine dihydrochloride to a colored product indophenol. when the growth of the organisim is rubbed over the filter paper containing the regent, a purple color is developed.

PROCEDURE

- A thin smear was made from the given culture.
- The smear was air dried and heat fixed.
- The smear was flooded with 5% malachite green for 6 minutes.
- The slide was kept on a water bath and steamed for 6 minutes.
- The preparation was gently washed with water.
- Finally the counter stain saffranin was added.
- The slide was washed and observed under microscope

CATALASE TEST

This tests for the bacteria's ability to splitting Hydrogen peroxide to oxygen and waterusing the enzyme catalase. If the organism has catalase it will split H_2O_2.

PROCEDURE

- Tube Method: Inoculate the test organism on agar slant and incubate for 24 hours. Allow 1 mL of 3% hydrogen peroxide to flow over the slant. B.
- Slide Method: Add one drop of 3% Hydrogen peroxide on a clean glass slide.Aseptically take a loopful of the test organism and emulsify in the H2O2drop.

RESULT

ENUMERATION OF BACTERIA FROM VEGETABLE

LISTERIA MONOCYTOGENES

A rod shaped gram positive bacterium were observed. Selectivemedia by streak plate.

MORPHOLOGICAL CHARACTERIZATION

To identify the organisms by using following test-Gram's staining, indole, MR, VP, citrate, urease, catalase, oxidase and carbohydrate fermentation test.

Bacterial contaminants were isolated from four different types of vegetables such as lettuce, cabbage, cucumber and carrots. By using selective Oxford agar media, the organisms are isolated. Typical bacterial colonies on the selective agar plates were subcultured on nutrientagar plates. Additional identification was accomplished by a series of tests to confirm morphological and biochemical characteristics. L.monocytogenes is a gram-positive, glucose, encapsulated, glucose fermenting, facultative anaerobic, Urease negative, oxidase negative, catalase postive organism belonging to the eubacteria family. The *Listeria monocytogenes* wasidentified by gram's staining, colony characteristics and biochemical tests such as catalase test,Urease test, Indole test, H2S test, motility, citrate test, oxidase disc test, VP test, Methyl red test. It's possible that brocolli, cauliflower and tomatoes are less contaminated than other vegetables they have less soil contact. The results of this study mainly compared the microbial populations and presence of *listeria* spp. In selected vegetables from four markets. Each type of vegetables purchased from the selected shops did not show significant differences in microbial quality. However, slight difference were observed. For cabbage samples, the Highest *listeria* spp. Loads were in samples from the market C, with a mean of 3.26 log cfu/g, while the lowest population of *listeria* were detected in the samples from market A, with a mean of

1.51 log cfu/g. Carrot samples had the lowest *listeria* spp. Load. Among four types of vegetables from four different shops, the highest were in market B(1.02 log cfu/g) and all the samples from market D showed negative results. The population of *listeria monocytogenes* in cabbage was similar to that in cucumber, was the highest value of *listeria* sp. Load in market A(3.2 log cfu/g) and the lowest value of *listeria* spp. Load in market B (1.52 log cfu/g).The lettuce samples had the highest bacterial load among the 4 types of vegetables and *listeria* spp.Levels **(Qi Zhu *et al*.,2016).** Most recent listeriosis outbreaks associated with fresh produce vegetables are attributed to the crop growing environment, post-harvest processing and retailing. The *listeria monocytogenes* could be detected in 22.5% of the vegetables. This is due to lack of hygiene, environmental contamination and vegetable handlers. To improve the fresh produce safety and preserve consumer health, it is important to lower the level of this pathogen.Using a practical and effective method to prevent the formation of *L.monocytogenes* , biofilmswill help to reduce the bacterial survival and contamination levels on fresh produce vegetables.

Table 1: *Listeria* contamination in vegetables

Sample	*L.monocytogenes*	*E.coli*
Lettuce	+	+
Cabbage	+	+
Cucumber	+	+
Carrot	+	+

Table 2: Biochemical test

S.NO	TEST	RESULT
1	Gram staining	Positive
2	Carbohydrate fermentation –mannitol	Negative
3	Rhamnose	Positive
4	Xylose	Negative
5	Indole	Negative
6	Methyl red	Positive
7	Voges –proskauer	Positive
8	Citrate	Negative
9	Urease production	Negative
10	Motility	Tumbling
11	Oxidase	Negative
12	Catalase	Positive

***LISTERIA* ON LETTUCE**

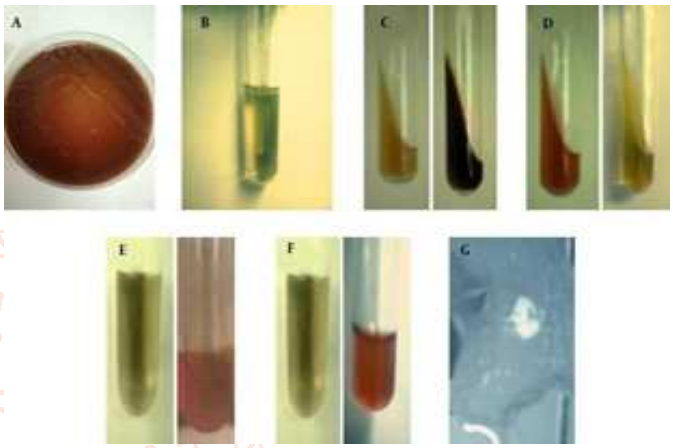
NEWLY CUT VEGETABLES

***LISTERIA* ON OXFORD AGAR**

***LISTERIA on* HBA PLATE**

***LISTERIA* IN PRIMARY ENRICHMENT BROTH**

BIOCHEMICAL TEST OF *LISTERIA*



CONCLUSION

The result of this study shows that the newly cutted vegetables contain *Listeria* were isolated from different markets indicating the lack of hygiene, environment contamination. However, the contamination of *listeria* spp. In lettuce was higher than in cabbage. The total microbial load in carrot samples was higher than in the cucumber samples. *Listeria* can be killed by cooking and pasteurization. But on raw fruits and vegetables, processing plants are required to prevent bacterial contamination with special washes and coldstorage.

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