Rapid detection of preexisting internal *Leuconostoc spp.* spoilage populations in fresh-cut carrots during storage

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Abstract

During an extended period of abnormally short quality retention in mixed component packaged salads, due primarily to rapid decay, a root-cause investigation was undertaken. From an initial investigative assessment, this study focused on identification for the primary underlying microbiological cause and validation of a rapid detection screening of raw material. Analysis of several lots of raw, unprocessed product, approx. 6 cm abrasively peeled carrot plugs, revealed the accumulation of an aqueous slime in the shipping bag void space and around the extremely softened plug surfaces. This premature diagnostic sign of lactic acid bacteria (LAB) spoilage, specifically *Leuconostoc spp.*, developed in cold storage (2.5°C) after two weeks. Efforts were undertaken to determine whether the *Leuconostoc* was internalized in raw material or primarily environmental contamination with a proliferating reservoir of LAB in the primary processing and packaging environment. Polymerase chain reaction (PCR) primers specific for the amplification of a sequenced region of the *Leuconostoc* 16S ribosomal RNA gene confirmed the taxonomic identity. Total initial LAB and *Leuconostoc* bacterial populations isolated from symptomatic carrots ranged from $\log_{10}$ 7.5–8.5 and $\log_{10}$ 3.5–4.0 CFU/g carrot tissue weight respectively, and increased $\log_{10}$ 2 CFU/g $\log_{10}$ and 3.5 CFU/g respectively in population density on asymptomatic and symptomatic raw carrot material during two week refrigerated storage.

Introduction

Although Lactic acid bacteria (LAB) are usually utilized extensively in dairy technology and for commercial fermentation of vegetables, such as carrots and cabbage, indigenous and naturally present LAB can also cause detrimental decay and spoilage on the raw product material if population growth becomes unmanageable (Gardner *et. al.*, 2001). This prolific population growth can result from processing failures involving neglected sanitation protocols and abusive postharvest storage conditions, which ultimately lead to the proliferation of spoilage LAB in the surrounding environment. Such rampant microbial growth can also initiate product spoilage before the "sell-by" or "use-by" date in grocery stores, amounting to considerable economic loss and marred commercial reputation amongst consumers. This troublesome form of decay of the raw material product drastically shortens the microbiological shelf-life and aesthetic quality of fresh-cut produce during storage and after packaging, especially when abusive storage conditions persist and weaken the physiology of the fresh-cut product..

Rapid and accurate detection of LAB contamination sites during the manufacture of fresh-cut produce are necessary to quickly prevent or target reservoir accumulation and overprovision of spoilage LAB. Environmental LAB spoilage microbes of fresh-cut processing can potentially derive from residual puddles of water or unclean liquid run-off as sources of contamination. From samples taken, isolated populations of LAB will most likely manifest. For further identification into the genus *Leuconostoc* and species *Leuconostoc mesenteroides*, several other traditional biochemical assays are used, but have slowly been replaced by faster and more accurate genomic tests, such as those included in this study. With the intention of rapidly diagnosing the microbial cause of this real-world postharvest processing concern, a rapid genetic detection method was developed for future utilization in commercial and industrial screening purposes of LAB populations as potential problems in postharvest storage of minimally processed carrot.

Materials and Methods

Four groups of raw product samples from CA fresh-cut facility were tested, each three months apart.

Symptomatic (final product) samples

Group 1: Four 12 oz. bags of company's salad mixture of iceberg lettuce, cabbage, and carrot vegetables; Four 6 oz. bags of shredded carrots used for integration into salad mixture; 2 kg of raw asymptomatic carrot plug material used for the shredded carrots.

Asymptomatic (raw product carrot material) samples

Group 2: Five 1.5 kg samples of raw carrot plug material labeled by "Lot" and product number. Group 3: Three 1.5 kg samples of raw carrot plug material. Group 4: Two different processed forms of the raw asymptomatic carrot plug material- One 1.5 kg sample of peeled raw product (from throughout processing storage bin), one 1.5 kg sample of unpeeled raw product (from top of bin), One 1.5 kg sample of unpeeled (from bottom of bin).

Isolation and investigation of LAB from all groups of samples

50 g of each salad mixture sample and shredded carrot sample stomached separately with 250 ml of Butterfield's Phosphate Buffer (BPB) in a Stomacher 400 at top speed for 60 seconds. One milliliter of the liquid salad mix and liquid carrot mix was removed from the stomached mixture and serially diluted to 10^{-5}. 100 µl of each dilution was plated onto MRS agar for lactic acid bacteria quantification, and 5% sucrose-amended Luria-Burtani (LB) agar for *Leuconostoc spp.* quantification and isolation. Plates were incubated at 37°C for 48 hours. For raw product, several independent replications of carrot plugs were surface-peeled with a sterile vegetable peeler, accrued approximately fifty grams in total, and was stomached with 100 ml of BPB in a Stomacher 400 at top speed for 60 seconds. Same plating and quantification technique were used.

Stimulating low levels of LAB and *Leuconostoc* populations by abusive storage conditions for raw carrot product using free moisture

After initial quantification of LAB and *Leuconostoc* populations in symptomatic samples, low levels suggested continual recontamination of raw product material with latent but inducible spoilage potential. 50 g of each sample were quickly dipped in sterile, nanopure water to increase free moisture levels, and placed at progressively higher storage temperatures 5°C, 7.5°C, 10°C, 15°C, and 20°C, replicating hypothesized spoilage conducive treatment during processing, storage, and transportation.

Identification of bacterial nternalization of raw carrot plugs

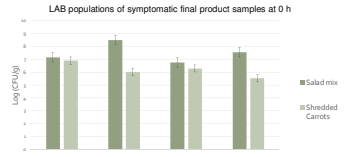
Samples of Group 4 asymptomatic unpeeled and peeled carrot plugs were treated with either sterilized water or 7.5% H_2O_2 and stored at 5°C for two weeks. Observations were made of possible *Leuconostoc spp.* internalization based on the development of slime and breakdown of internal tissue.

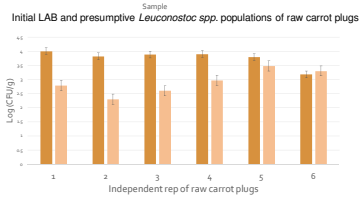
DNA extraction and PCR assays for molecular and diagnostic identification of *Leuconostoc spp.*

Leuconostoc-specific primers amplifying a 168 bp region were created based on aligning the sequences of the genes encoding the 16S rRNA of *Ln. mesenteroides* subsp. *mesenteroides*, *Weissella viridescens*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus sake*, and *Lactobacillus acidophilus* (Goto et. al., 2004). Following culture confirmation and phenotypic isolation of LAB and *Leuconostoc spp.* colonies, DNA was extracted from each LAB isolate that showed phenotypic characteristic of diagnostic leuconostocs using a modified version of the method from Chun & Han (2000), and used as template DNA for the PCR.

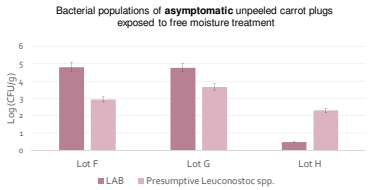
Results

Group 1





Group 3



Group 2

Sample	LAB populations of asymptomatic raw carrot plugs at T=0 (all in CFU/g)	LAB after exposure to free moisture treatment	Presumptive *Leuconostoc spp.* populations of asymptomatic raw carrot plugs at T=0	Presumptive *Leuconostoc spp.* after exposure to free moisture treatment
Lot A	1.6	2.3	1.3	3.04
Lot B	2.25	2	2.47	3
Lot C	2.47	1.9	1.9	2.81
Lot D	1.6	1.3	2	3.15
Lot E	3.47	3.3	2.3	3.11

Group 4

Unpeeled raw carrot samples

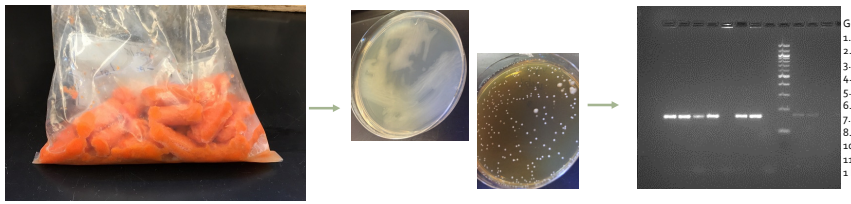
Exposure to abusive storage conditions	LAB enumeration Log(CFU/g)	Presumptive *Leuconostoc spp.* enumeration Log(CFU/g)
Incubation at 5°C for 7 days, then 10°C for 7 days	1.2	0
Incubation at 7.5°C for 7 days, then 15°C for 7 days	0	0
Incubation at 10°C for 7 days, then 20°C for 7 days	7.9	4.1

Peeled raw carrot samples

Exposure to abusive storage conditions	LAB enumeration Log(CFU/g)	Presumptive *Leuconostoc spp.* enumeration Log(CFU/g)
Incubation at 5°C for 7 days, then 10°C for 7 days	0	0
Incubation at 7.5°C for 7 days, then 15°C for 7 days	3.5	0
Incubation at 10°C for 7 days, then 20°C for 7 days	7.9	4.3

Conclusions

This study was prompted by the observation of diagnostic spoilage signs and symptoms on raw, fresh-cut carrots in storage, indicative of proliferative populations of LAB, specifically *Leuconostoc.* In order to identify and verify the major microbial culprit behind this spoilage, 4 independent samplings were performed and further aimed to improve the efficiency for analysis and rapid screening of symptomatic carrot plug lots. Testing for rapid LAB identification used, including PCR, was to amplify the presence of a region of the 16S rRNA gene of *Leuconostoc spp.* Previous tests for identifying spoilage organisms like LAB have included cumbersome biochemical tests requiring days to weeks for adequate and diagnostic results. After sampling symptomatic raw carrot plugs, asymptomatic plugs, and carrot plugs of different peeled treatments, *Leuconostoc spp.*, the notorious spoilage microbe of raw carrots, was discovered for all treatments. Common microbiological techniques were used to quantify and identify overall LAB and *Leuconostoc spp.* populations, and PCR confirmed the presence of *Leuconostoc spp.*, offering a rapid screening of symptomatic or asymptomatic carrot tissue for early warning of potential spoilage in storage.



Gel showing 168 bp amplified sequence
1. Lettuce mix sample 1
2. Lettuce mix sample 2
3. Lettuce mix sample 3
4. Lettuce mix sample 4
5. Lot F
6. Lot G
7. *L. mesenteroides* ATCC 10881
8. *Lactobacillus plantarum*
10. Lot A
11. Lot B
1 kb ladder used