Microbial Evaluation of Fresh Marketed Vegetables

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Abstract

Fresh marketed vegetables were analyzed for bacterial flora. The count of total bacteria and coliform group ranged from 4.5 to 6.5 log colony-forming units (CFU) /g and 3.3 to 4.3 log CFU/g, respectively with cucumber, lettuce, spinach and carrots, while bacterial count was below the detection level (2.4 log CFU/g) with tomatoes, onions and garlic. Cucumber, lettuce and carrots had approximately 1 to 3 logs higher bacterial levels in outer tissue than in inner tissue, whereas spinach had similar bacterial levels on the exterior and interior leaves. Gram-negative isolates were the predominant bacteria with carrots and spinach, while the proportion of Gram-positive and Gram-negative isolates were almost equal with cucumber. The frequently isolated genera from carrots, spinach and cucumber were *Curtobacterium*, *Staphylococcus* and *Enterococcus* as Gram-positive and *Pseudomonas*, *Agrobacterium*, *Stenotrophomonas*, *Enterobacter*, *Pantoea* and *Klebsiella* as Gram-negative. No human pathogens were detected in any of the vegetables tested.

1. Introduction

High quality fresh fruits and vegetables are now available because of advanced technology developed from research on cultural practices, harvesting, packaging and distribution. However, information is lacking on microbial quality and safety of fresh produce in relation to some of the advanced technologies in Japan. Within the past decade, the number of produce-associated foodborne disease outbreaks increased significantly in the US [1, 2, 3, 4]. These outbreaks linked *Escherichia coli* O157:H7 with apple cider/juice, lettuce, bean sprouts and broccoli, *Clostridium botulinum* with garlic, *Salmonella* spp. with watermelon, cantaloupe, tomatoes and bean sprouts, *Shigella* spp. with green onions and lettuce and *Listeria monocytogenes* with lettuce, cabbage and carrots. The US Food and Drug Administration (FDA) initiated a 1000 sample survey to determine the incidence and extent of human pathogens such as *Shigella*, *Salmonella* and *E. coli* O157:H7 on imported fresh produce and domestic fresh produce in 2000. The FDA announced the discovery of either *Shigella* or *Salmonella* in 44 of 1003 imported produce samples (4% of the total number sampled) including cantaloupe, cilantro and culantro [5] and in 11 of 1028 domestic produce samples (1% of the total number sampled) including cantaloupe, green onions and lettuce [6]. *E. coli* O157:H7 was not found on any of the produce samples.

In 1998, the FDA released voluntary guidelines entitled "Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" that outlines good agricultural practices (GAPs) and good manufacturing practices (GMPs) to reduce the risk of microbial contamination of fresh produce [7]. GAPs and GMPs have been recommended on all stages of the farm-to-tables food chain and practically implemented by growers, packers and shippers in the US. In Japan, proactive and practical programs such as GAPs and GMPs were not developed due to insufficient data on microbiological quality assessment on fresh produce, although Japan Greenhouse

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Horticulture Association is preparing guidelines for vegetables grown in soil culture.

Our objective was to investigate the bacterial flora of vegetables selected from supermarket to conduct effective risk assessments of the microbial quality and hazards associated with fresh produce in Japan. Microbial standards for processed and lightly processed produce have been defined, whereas any regulations governing microbial contamination are not in place for fresh fruits and vegetables. Data from this study will provide a baseline to establish the new microbial standards on produce.

2. Materials and Methods

Samples of vegetables (cucumber, tomatoes, lettuce, spinach, carrots, onions and garlic) were purchased from 3 supermarkets in Wakayama in June for microbial counts. Each vegetable was aseptically separated into outer and inner tissues with sterile scalpels. The outer tissues were the epidermis and exocarp of cucumber, exocarp and mesocarp of tomatoes, exterior leaves of lettuce and spinach, cortex and endodermis of carrots and exterior scaly leaves of onions and garlic. The inner tissues were the placenta of cucumber and tomatoes, interior leaves of lettuce and spinach, xylem of carrots and interior scaly leaves of onions and garlic. Each macerated sample was assayed in at least triplicate for total bacterial count on standard method agar (Nissui Pharmaceutical Co. Ltd, Tokyo) and for coliforms on desoxycholate agar (Nissui Pharmaceutical Co. Ltd, Tokyo) as previously described [8]. Microbial counts were expressed as $\log_{10}$ colony-forming units (CFU) / g sample.

Fresh carrots, cucumber and spinach were obtained from 3 supermarkets in July, October or December for identification of bacteria on outer and inner tissues. Different colonies by a stereoscopic observation were isolated from EOA agar (Nissui Pharmaceutical Co. Ltd, Tokyo) plate for Gram-positive bacteria and CVT agar (Nissui Pharmaceutical Co. Ltd, Tokyo) plate for Gram-negative bacteria inoculated with the proper dilutions of the homogenates of the vegetable samples. Pure cultures of bacteria were classified according to their cellular morphology observed by microscopy, Gram stain, oxidase test and thermostable test for spore-former [9], and identified using the Biolog microstation system in accordance to the protocol (GSI Cress, Inc., Tokyo). Cultures to be tested were subcultured on BUG agar containing 0.25% maltose and 0.9% thioglycolate for spore-forming *Bacillus* spp. or the agar containing 5% sheep blood for other strains, and then incubated at 30-35℃. A homogeneous suspension of inoculum was made in 0.85% saline and diluted to the designated transmittance ranging from 20% to 63%. One hundred fifty μl of suspension was dispensed into each well containing 95 different carbon source and a control well with tetrazolium violet of the GN or GP Microlog microplate, which was incubated for 24h at 35℃. Microplates were read at 590 nm at 4 and 24 h with a MicroPlate reader. A tetrazolium-based color change based on the exchange of electrons produced during respiration of organism was compared automatically with the database including 1214 species. Only reports with a probability index of >80% were considered an acceptable genus and species identification. The Biolog reported identifications for 266 of 352 organisms tested (75.6%), and of the 266 identifications, 87.3% were correct at the genus level and 75.6% were correct at the species level [10].

3. Results

The count of total bacteria and coliform group from countable plates ranged from 3.0 to 6.5 log CFU/g and 3.3 to 4.3 log CFU/g, respectively with cucumber, lettuce, spinach and carrots, while bacterial count was below the detection level (2.4 log CFU/g) with tomatoes, onions and garlic (Table 1). Cucumber contained the highest counts of total bacteria and coliforms of any vegetables. Cucumber, lettuce and carrots had approximately 1 to 3 logs higher bacterial levels in outer tissues than in inner tissues, whereas spinach had similar bacterial levels on the exterior and interior leaves, possibly due to the alternate phyllotaxis of spinach exposing all leaves equally for environmental contamination.

Table 1 Counts of total bacteria and coliform group from outer and inner tissues of several vegetables

Vegetable	Tissue	Log CFU/g	
		Total bacteria	Coliform group
Cucumber	Outer	6.5	4.3
	Inner	3.5	<2.4 [Z]
		**	**
Tomato	Outer	<2.4	ND [Y]
	Inner	ND	ND
Lettuce	Outer	4.7	3.3
	Inner	3.0	<2.4
		**	**
Spinach	Outer	4.5	3.9
	Inner	4.9	3.9
		NS	NS
Carrot	Outer	5.4	3.3
	Inner	<2.4	ND
		**	**
Onion	Outer	ND	ND
	Inner	ND	ND
Garlic	Outer	<2.4	ND
	Inner	<2.4	ND

[Z] Below the detection level (2.4 log CFU/g).
[Y] Not detectable.
NS, **: Nonsignificant or significant at 1% level, respectively, between paired outer and inner tissues within total bacteria or coliform group.

The genera of bacteria were similar in isolates from carrots harvested in July and December and the majority of organisms were Gram-negative bacteria belonging to the genera *Pseudomonas*, *Pantoea* and *Enterobacter* (Table 2 and Table 3). About 60% of isolates were Enterobacteriaceae including the genera *Pantoea*, *Enterobacter*, *Klebsiella*, *Escherichia*, *Serratia*, *Cedecea* and *Buttiauxella* and some species of isolates were plant pathogenic bacteria such as *Agrobacterium rhizogenes*, *Pseudomonas cichorii* and *fluorescens*, *Pantoea agglomerans* and *Stenotrophomonas maltophilia*. Isolated *Enterococcus casseliflavus* in Gram-positive and *Klebsiella pneumoniae* and *Escherichia vulneris* in Gram-negative may be related to fecal origin.

The proportion of Gram-positive and Gram-negative bacteria was similar in isolates from cucumber in July (Table 4). Majority of Gram-positive isolates were the genus *Staphylococcus* and lactic acid bacteria such as *Pediococcus urinaeequi* or *Enterococcus casseliflavus*, and the Gram-negative isolates were Enterobacteriaceae including the genera *Pantoea*, *Enterobacter* and *Klebsiella* and plant

Table 2 Bacteria isolated from carrots obtained from a supermarket in July

Gram type	Bacteria	
	Genus	Species/Subspecies
Gram-positive	*Leuconostoc*	*mesenteroides* subsp. *mesenteroides*
	Curtobacterium	*pusillum*
Gram-negative	*Agrobacterium*	*rhizogenes*
	Pseudomonas	*cichorii*
	Pseudomonas	*fulva*
	Pantoea	*agglomerans*
	Enterobacter	*amnigenus*
	Enterobacter	*asburiae*
	Enterobacter	*cloacae*
	Enterobacter	*nimipressuralis*
	Klebsiella	*pneumoniae* subsp. *pneumoniae*
	Escherichia	*vulneris*

Table 3 Bacteria isolated from carrots obtained from a supermarket in December

Gram type	Bacteria	
	Genus	Species/Subspecies
Gram-positive	*Lactococcus*	*lactis* subsp. *lactis*
	Enterococcus	*casseliflavus*
Gram-negative	*Pseudomonas*	*fluorescens*
	Stenotrophomonas	*maltophilia*
	Pantoea	*agglomerans*
	Pantoea	*dispersa*
	Enterobacter	*amnigenus*
	Enterobacter	*cloacae*
	Serratia	*fonticola*
	Cedecea	*lapagei*
	Buttiauxella	*gaviniae*

Table 4 Bacteria isolated from cucumber obtained from a supermarket in July

Gram type	Bacteria	
	Genus	Species/Subspecies
Gram-positive	*Pediococcus*	*urinaeequi*
	Enterococcus	*casseliflavus*
	Curtobacterium	*pusillum*
	Staphylococcus	*sciuri*
	Staphylococcus	*xylosus*
	Brochothrix	*thermosphacta*
Gram-negative	*Agrobacterium*	*tumefaciens*
	Pseudomonas	*fluorescens*
	Stenotrophomonas	*maltophilia*
	Pantoea	*agglomerans*
	Pantoea	*dispersa*
	Enterobacter	*asburiae*
	Klebsiella	*pneumoniae* subsp. *rhinoscleromatis*

pathogenic bacteria such as *Agrobacterium tumefaciens*, *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia* and *Pantoea agglomerans*. *Brochothrix thermosphacta*, a Gram-positive isolate, was detected in our sample and is known as meat spoilage bacteria.

The Gram-negative rod-forms were the predominant bacteria on spinach leaves in October, making up over 70% of the isolates (Table 5). The numerous species were member of Enterobacteriaceae such as the genera *Pantoea*, *Enterobacter* and *Citrobacter* or plant pathogenic bacteria belonging to the genera *Agrobacterium*, *Pseudomonas*, *Stenotrophomonas* and *Pantoea*, in which *Enterobacter cloacae* and *Pseudomonas aeruginosa* were typical opportunistic pathogens.

Table 5 Bacteria isolated from spinach obtained from a supermarket in October

Gram type	Bacteria	
	Genus	Species
Gram-positive	*Curtobacterium*	*pusillum*
	Staphylococcus	*sciuri*
	Bacillus	*thermoglucosidasius*
Gram-negative	*Agrobacterium*	*tumefaciens*
	Pseudomonas	*aeruginosa*
	Pseudomonas	*putida*
	Stenotrophomonas	*maltophilia*
	Pantoea	*agglomerans*
	Pantoea	*dispersa*
	Enterobacter	*cloacae*
	Citrobacter	*freundii*

4. Discussion

Total bacterial counts on fresh vegetables, reported by different authors, varied widely within and among vegetables. Reported counts were 3.6 to 6.1 log CFU/g in lettuce [11,12], 6 to 7 log CFU/g in spinach [13], 2.1 to 4.7 log CFU/g in tomatoes [14,15], 5 to 6 log CFU/g in carrots [12], 3 to 4 log CFU/g in celery [16] and 6.5 log CFU/g in broccoli [17]. Bacterial population is affected by environmental and handling conditions and the conditions are not known with these reported counts, but these counts are similar to those observed in this study except for tomatoes, onions and garlic. Tomatoes had a pH of below 4 where most pathogens are inhibited, which may have contributed to the lower bacterial counts. Onions and garlic are generally cured and stored in low relative humidity (65 to 70%) [18]. This results in 3 to 5% water loss, which contributes to lowering of water activity and subsequently, possibly have an inhibitory effect on the growth of bacteria. In addition, the pungent volatile components such as diallyldisulfide in garlic [19] may have an antimicrobial effect as noted with allyl isothiocyanate extracted from mustard [20] and wasabi [19] and capsaicin from paprika [21].

Microbial contamination on vegetables occurs during production, harvest and distribution and the degree of contamination depends on the environmental conditions from growing to marketing and physiological condition of the product [22]. Bacterial count on the exterior tissues would be expected to be higher than that on interior tissues, because the exterior tissues are exposed more to environmental contamination than inner tissues as noted with sound cucumber, lettuce and carrots in this study and reported on lettuce microflora [23].

Types of bacteria found in carrots, cucumber and spinach in this study were similar to those found on broccoli [17], tomatoes [14,15], lettuce [11], celery [16] and spinach [13]. The proportion of Gram-positive and Gram-negative differed among vegetables. Gram-negative isolates were the predominant bacteria in carrots and spinach, while the proportion of Gram-positive and Gram-negative isolates were almost equal in cucumber. The frequently isolated genera from these vegetables were *Curtobacterium*, *Staphylococcus* and *Enterococcus* as Gram-positive and *Agrobacterium*, *Pseudomonas*, *Stenotrophomonas*, *Enterobacter*, *Pantoea* and *Klebsiella* as Gram-negative. These bacteria were found frequently in soil and stream water and do not usually represent a public health concern [24].

Although some opportunistic bacteria such as *Pseudomonas aeruginosa*, *Enterobacter amnigenus*, *Enterobacter cloacae* and *Serratia fonticola* were identified on all vegetables, no human pathogens were detected in any of the samples tested. However, detection of *Enterococcus casseliflavus* or *Escherichia vulneris* on carrots and *Klebsiella pneumoniae* on carrots and cucumber is of a concern, because presence of these microorganisms is correlated with fecal matter of warm blooded animals. When fecal coliform count exceeds 1000, there is a high percentage of *Salmonella* present in the stream water [25]. Since fecal contamination of fresh vegetables can be due to preharvest factors such as irrigation water, animal manure and human handling and/or postharvest factors such as wash water, human handling, equipments in storage or packaging and transportation vehicles [3,25], more attention must be given to careful handling and sanitary conditions from cultivation to marketing processes to prevent fecal contamination.

5. References

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和文抄録

市販野菜の微生物的評価

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市販野菜のバクテリアルフローラを調査した。キュウリ，レタス，ホウレンソウおよびニンジンの一般生菌数と大腸菌群数は，それぞれ 4.5～6.5logCFU／g と 3.3～4.3logCFU／g であるのに対して，トマト，タマネギおよびニンニクの細菌数は検出限界値（2.4logCFU／g）以下であった。キュウリ，レタスおよびニンジンでは，外部組織のほうが内部組織よりも対数値で１～３程度高い細菌数を示したが，ホウレンソウでは両組織間に差は見られなかった。ニンジンとホウレンソウでは，グラム陰性菌が主体をなしたのに対して，キュウリではグラム陽性菌とグラム陰性菌がほぼ同程度検出された。ニンジン，ホウレンソウおよびキュウリから頻繁に検出された細菌は，グラム陽性菌では *Curtobacterium* 属菌，*Staphylococcus* 属菌および *Enterococcus* 属菌で，グラム陰性菌では *Pseudomonas* 属菌，*Agrobacterium* 属菌，*Stenotrophomonas* 属菌，*Enterobacter* 属菌，*Pantoea* 属菌および *Klebsiella* 属菌であった。なお，いずれの野菜からも食性病原細菌は検出されなかった。