Influence of Soft Rot Bacteria on Growth of *Listeria monocytogenes* on Potato Tuber Slices†

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ABSTRACT

Growth of *Listeria monocytogenes* on potato tuber slices and its interaction with four representative species of soft rot bacteria (*Pseudomonas fluorescens, P. viridiflava, Erwinia carotovora* subsp. *carotovora,* and *Xanthomonas campestris*) were investigated. When potato tuber slices were inoculated with one of two *L. monocytogenes* strains (Scott A and ATCC 15313), an increase in numbers of 3 to 4 logs per gram of tissue was observed with samples that were stored at 20°C for 6 days. However, an increase of about 2 logs was observed with samples that were stored at 8°C for 12 days. When potato slices were simultaneously inoculated with *L. monocytogenes* and one of the four soft rot bacteria, the growth of *L. monocytogenes* was inhibited in the presence of *P. fluorescens* or *P. viridiflava* but was not significantly affected in the presence of *E. carotovora* or *X. campestris.* The antagonism of the two pseudomonads to *L. monocytogenes* was also observed in potato tuber extract and in culture media. Formation of inhibition zones was observed only in iron-deficient media but not in the medium supplemented with $FeCl_3$. In addition, production of fluorescent siderophore (pyoverdin) by these two pseudomonads was demonstrated. *L. monocytogenes* was unable to colonize macerated plant tissue induced by soft-rotting bacteria 2 days before inoculation of the pathogen. These results indicate that growth of *L. monocytogenes* on potato tuber slices is differentially affected by soft rot bacteria and that antagonism of fluorescent pseudomonads to *L. monocytogenes* is possibly caused by the production of iron-chelating siderophore by these pseudomonads.

Listeria monocytogenes, which causes a serious human illness generally known as listeriosis, has emerged as an important foodborne pathogen in the past two decades *(20).* Although foodborne listeriosis has been most commonly traced to consumption of dairy products, including milk and soft cheese *(20),* several studies *(11, 23)* have shown that raw vegetables also serve as a vehicle for pathogen transmission. Because of its ubiquitous nature, *L. monocytogenes* has been found frequently in fresh and refrigerated fruits and vegetables *(10, 16, 24).* For example, Sizmur and Walker *(24)* reported that 4 of 60 prepackaged, ready-to-eat salads studied were contaminated with *L. monocytogenes.* Hesick et al. *(10)* examined 1,000 samples of fresh produce in the United States and found that *L. monocytogenes* was present on cabbage, cucumber, potatoes, radishes, and other vegetables. Moreover, several inoculation studies *(2, 3, 5, 25)* have demonstrated the ability of *L. monocytogenes* to grow on fresh produce stored at various conditions. This pathogen thus represents a potentially serious hazard in fresh and minimally processed fruits and vegetables.

Despite its ecological importance, very little is known about the interaction kinetics between *L. monocytogenes* and resident microflora naturally present on plants. Carlin et al. *(6, 7)* recently reported that growth of *L. monocytogenes* on endive leaves was affected by several factors, including temperature, age of the leaves, and characteristics of the *L. monocytogenes* inoculum. Babic et al. *(1)* showed that growth of *L. monocytogenes* on fresh-cut spinach was restricted by some native microorganisms, including *Staphylococcus xylosus* and various biovars of *Pseudomonas fluorescens.* The quality and quantity of resident microflora are considered key factors that determine the fate of *L. monocytogenes* on fresh produce *(6).*

Soft-rotting bacteria, including *Erwinia, Pseudomonas, Xanthomonas,* and *Cytophaga,* constitute an important part of resident microflora on fresh produce and are responsible for a substantial proportion of spoilage in fresh and minimally processed vegetables *(14, 18, 19).* Carlin et al. *(6)* recently reported a positive correlation between the increase in numbers of *L. monocytogenes* and the increase in numbers of aerobic bacteria and the extent of spoilage on endive leaves. Wells and Butterfield *(26)* reported that *Salmonella* contamination was more often associated with soft-rotted tissue than with the healthy part of plants. These two studies suggested that soft-rotted tissue may serve as a site for the growth of human pathogens and represents a warning sign for possible contamination by enteric bacteria *(26).* A better understanding of the interactions between soft rot bacteria and human pathogens would provide a basis for the development of new strategies for improving the quality and safety of fresh produce. The purpose of this study was

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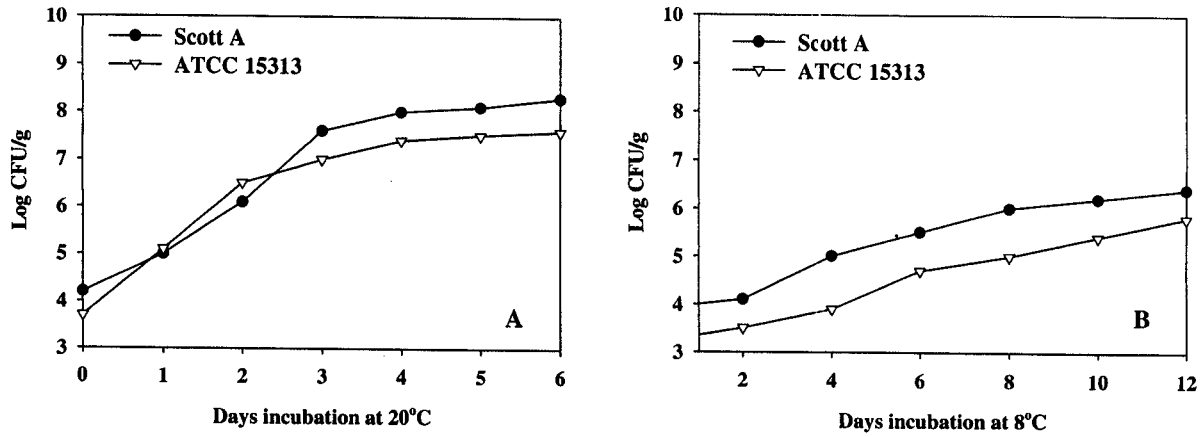

FIGURE 1. *Changes in number of* L. monocytogenes *Scott A and ATCC 15313 on fresh-cut potato tuber slices stored at 20°C (A) and 8°C (B).*

to investigate changes in *L. monocytogenes* counts and its interactions with four representative species of soft rot bacteria on potato tuber slices.

MATERIALS AND METHODS

Bacterial strains and selective media. Bacterial strains used in this study included two wild-type strains of *L. monocytogenes* (Scott A and ATTC 15313), four rifampicin-resistant strains of soft rot bacteria (*P. fluorescens* CY091A, *P. viridiflava* PJ-08-6A, *E. carotovora* subsp. *carotovora* SR319A, and *X. campestris* XC5A) *(14)* and a *gacA*$^-$ mutant of *P. fluorescens* CY091A deficient in production of fluorescent siderophore *(15)*. All these bacterial strains were obtained from our own culture collection. *Pseudomonas* strains were routinely maintained on *Pseudomonas* agar F (Difco Laboratories, Detroit, Mich.), whereas *L. monocytogenes* strains were maintained on brain-heart infusion agar (Difco). When liquid media were required, *L. monocytogenes* and soft-rotting bacteria were all grown in brain-heart infusion broth. The cultures were incubated at 28°C unless otherwise indicated. To determine the numbers of *L. monocytogenes* and soft rot bacteria on potato tuber slices or in potato tuber tissue extract, two selective agar media were used. Modified Vogel Johnson (MVJ) medium *(4)* was used to determine the numbers of *L. monocytogenes*, and *Pseudomonas* agar F (PAF) containing 100 μg/ml rifampicin was used to determine the number of soft rot bacteria.

Preparation and inoculation of fresh-cut potato tuber slices. Potato tubers (Russet Burbank) obtained from local stores were peeled and surface-sanitized for 10 min in 1% sodium hypochlorite. After rinsing in sterile water, potato slices (18 mm in diameter and 3 mm in depth) were prepared as previously described *(14)*. Potato slices were inoculated with a bacterial suspension to contain approximately 10^4 to 10^5 CFU per gram of tissue and then placed on soft-water agar plates (0.6% agar in distilled water; three disks per plate). For preparation of bacterial suspensions, bacterial growth on agar media was harvested and resuspended in phosphate-buffered saline (PBS, pH 7.2; Life Technologies, Gaithersburg, Md.) to make a concentration of approximately 10^7 to 10^8 CFU/ml. Inoculations were made by dipping the potato slices in the bacterial suspension for 20 ss and then blotting them dry on paper towels. During the course of incubation at the temperature specified, potato slices were periodically removed and then suspended in 80 to 100 ml of peptone water. Samples were homogenized using a Stomacher (model 400, Seward Med., Ltd., London, UK), serially diluted, and plated onto MVJ and PAF-rifampicin to select *L. monocytogenes* and soft rot bacteria, respectively. All experiments were done in triplicate, and a minimum of two samples was analyzed at each sample time. Analysis of variance and Duncan's multiple-range test (SAS Institute, Inc., Cary, N.C.) were performed to determine significant differences on the logarithm (base 10) of bacterial population densities *(21)*.

Preparation of potato tuber tissue extract. Five hundred grams of peeled and surface-sterilized potato tuber slices were mixed with 500 ml of PBS and homogenized by using a Waring blender. The homogenate was filtered through four layers of cheesecloth. The filtrate was centrifuged, and the supernatant was sterilized by autoclaving (121°C, 15 min). The extract was inoculated with *L. monocytogenes* or a mixture of *L. monocytogenes* and a soft rot bacterium at initial concentration of approximately 10^5 CFU/ml for each organism. Changes in number of bacteria were monitored as described above, and production of pyoverdin by fluorescent pseudomonads in the extract was determined using the spectrophotometric method described below.

Assays of siderophore production. For assays of siderophore production, bacterial strains were each grown on chrome azurol S (CAS) agar at 28°C for 2 days *(22)*. CAS was purchased from Sigma Chemical Co. (St. Louis, Mo.). Production of siderophores, as indicated by formation of an orange zone surrounding the area of bacterial growth, was determined spectrophotometrically by measuring the size (diameter, in mm) of the orange zone. Alternatively, bacteria were grown in potato tuber tissue extracts or liquid PAF medium, and the concentration of fluorescent pyoverdin in the culture supernatant was measured using a spectrophotometer at 400 nm. The molecular extinction coefficient of 2×10^4 M^{-1} cm^{-1} was used to determine the quantity of pyoverdin present *(12)*.

RESULTS

Growth of *L. monocytogenes* on potato tuber slices. Surface-sanitized potato tuber slices were inoculated with one of two *L. monocytogenes* strains (Scott A and ATCC 15313), and changes in numbers of *L. monocytogenes* on potato slices stored at 8 and 20°C were monitored for up to 12 days. Figure 1 shows that both Scott A and ATCC 15313 are capable of growing on potato tuber slices at about the same rate. After 6 days of storage at 20°C, *L. monocytogenes* populations increased 3 to 4 logs (Fig. 1A), whereas increases of only about 2 logs were observed when

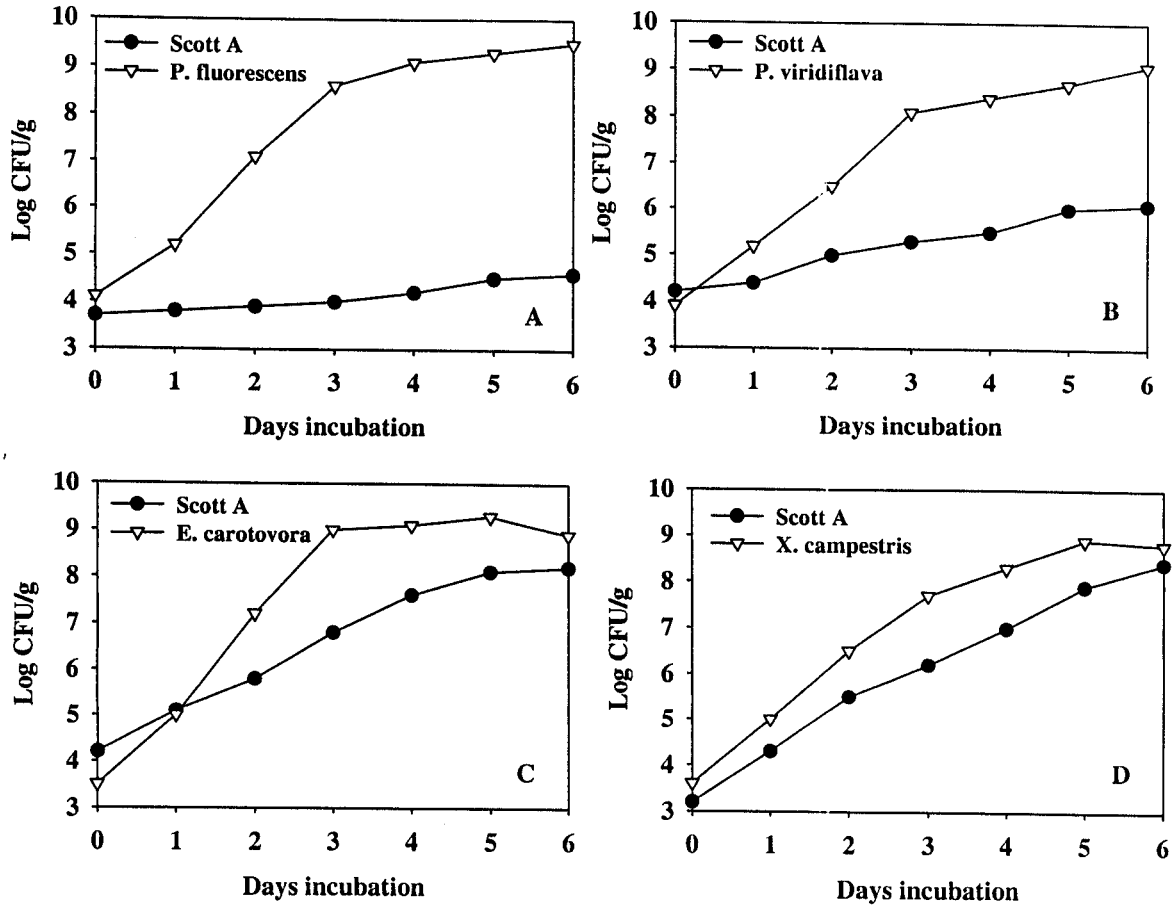

FIGURE 2. *Changes in number of* L. monocytogenes *Scott A on potato tuber slices simultaneously inoculated with one of four soft rot bacteria: (A)* P. fluorescens *CY091A, (B)* P. viridiflava *PJ-08-6A, (C)* E. carotovora *subsp.* carotovora *SR319A, or (D)* X. campestris *XC-5A.*

samples were stored at 8°C for 12 days (Fig. 1B). No indication of microbial growth, as determined by directly plating potato tuber tissue homogenates onto brain-heart infusion agar or PAF media, was observed on uninoculated potato tuber slices. This result indicates that *L. monocytogenes* is capable of growing on potato tuber slices to fairly high titers even in the absence of native microflora naturally present on the surface of plants.

Inhibitory effect of soft-rotting pseudomonads on *L. monocytogenes*. To investigate the effect of soft rot bacteria on the survival and growth of *L. monocytogenes* on plants, potato tuber slices were simultaneously inoculated with *L. monocytogenes* and one of the four soft rot bacteria. Changes in numbers of *L. monocytogenes* and soft rot bacteria were monitored daily for 6 days using MVJ and PAF-rifampicin medium, respectively. Results (Fig. 2) show that growth of *L. monocytogenes* on potato tuber slices was almost completely inhibited in the presence of *P. fluorescens* or *P. viridiflava* but not significantly affected in the presence of *E. carotovora* and *X. campestris.* In potato tuber slices that were inoculated with a mixture of *L. monocytogenes* and *P. fluorescens* (or *P. viridiflava*), both pseudomonads grew normally and reached a final concentration of more than 9 logs per gram of tissue, but an increase of only 0.9 to 1.9 logs was observed with *L. monocytogenes,* in contrast to the 3- to 4-log increase observed in the absence of pseudomonads. However, in potato tuber slices that were inoculated with a mixture of *L. monocytogenes* and *E. carotovora* (or *X. campestris*), both *L. monocytogenes* and *E. carotovora* (or *X. campestris*) grew equally well on potato tuber slices and reached final concentrations of 10^8 CFU per gram of tissue or higher. The concentration of *L. monocytogenes* was only slightly (about 1 log) lower than the number of *E. carotovora* or *X. campestris* (Fig. 2). This result indicates that growth of *L. monocytogenes* on potato tuber slices is differentially affected by soft rot bacteria, which can either be inhibitory or have no effect.

Inability of *L. monocytogenes* to colonize soft-rotted tissue. To determine whether the soft-rotted tissue might potentially serve as a site for growth of *L. monocytogenes,* potato tuber slices were first inoculated with one of the four soft-rotting bacteria and then incubated at 20°C for 2 days to allow development of soft rot. After that, macerated tissue was challenged with *L. monocytogenes* Scott A, and the initial and final numbers of *L. monocytogenes* after 5 days of incubation at 20°C were determined. As shown in Table 1, very little or no growth of *L. monocytogenes* was observed in tissue containing soft rot induced by *P. fluorescens* or *P. viridiflava.* However, an increase (1.5 to 1.8 logs) in the number of *L. monocytogenes* was observed in soft-

TABLE 1. *Changes in number of* L. monocytogenes *Scott A on macerated potato tuber slices induced by one of four soft rot bacteria*

Bacteria	Number of *L. monocytogenes* (log CFU/g)[a]	
	Initial concentration	Final concentration
P. fluorescens CY091A	4.25 ± 0.24	4.13 ± 0.14
P. viridiflava PJ-08-6A	3.81 ± 0.12	4.01 ± 0.20
E. carotovora subsp. *carotovora* SR319A	5.15 ± 0.31	6.98 ± 0.30*
X. campestris XC-5A	3.02 ± 0.05	4.52 ± 0.20*

[a] Values represent the average of three replicates ± standard deviation. Values with an asterisk were significantly different from the initial number of cells ($P \leq 0.05$).

rotted tissue induced by *E. carotovora* or *X. campestris.* This result indicates that *L. monocytogenes* did not colonize or colonized very poorly in the macerated tissue induced by soft rot bacteria 2 days before inoculation of the pathogen.

Relation of the antagonistic action to fluorescent siderophore production. Because growth of *L. monocytogenes* was inhibited only by the two fluorescent pseudomonads, it was suspected that inhibition possibly resulted from production of fluorescent siderophores by these two pseudomonads. To verify this, all four soft rot bacteria were first grown in various agar media (Table 2) at 28°C for 60 h to allow production of antimicrobial compounds and then killed by exposing them to drops of chloroform placed inside the cover of the petri dish for 1 min. After that, agar plates were overlaid with 3 ml of soft-water agar (0.6% agar in distilled water) containing approximately 10^5 CFU per ml of *L. monocytogenes* Scott A, and incubation was continued for an additional 3 days. Growth inhibition of *L monocytogenes,* as indicated by formation of clear zones surrounding the growth of soft rot pseudomonads, was observed (Fig. 3). Inhibition zones of various sizes were observed in iron-deficient PAF plates that had been preinoculated with *P. fluorescens* or *P. viridiflava* (Table 2) but were not found or were greatly reduced in PAF plates supplemented with 1 mm of $FeCl_3$. *P. fluorescens* and *P. viridiflava* grown in nutrient agar had no effect on the growth of *L. monocytogenes.* However, pseudomonads grown on potato dextrose agar (Difco) and in potato tuber tissue extract exhibited an inhibitory effect on *L. monocytogenes.* In contrast, *E. carotovora* and *X. campestris* had little or no effect on the growth of *L. monocytogenes* when grown together in culture media or in potato tuber tissue extract. In addition, a *P. fluorescens gacA*$^-$ mutant that was deficient in siderophore production *(15)* was also unable to inhibit growth of *L. monocytogenes* in iron-deficient PAF media and potato tuber tissue extract. When assayed on CAS agar plates specific for the detection of metal-chelating siderophore *(22),* the two pseudomonads formed the distinctive orange zones (5 to 12 mm in diameter) in agar plates. Production of pyoverdin siderophore by the two pseudomonads in potato tuber tissue extracts in the range of 7.8 to 8.5 mM per 10^{10} cells was further confirmed by the spectrophotometric method previously described *(12).* Growth of *L. monocytogenes* in potato tuber tissue extract was also inhibited in the presence of *P. fluorescens* or *P. viridiflava* but not markedly affected in the presence of *E. carotovora* or *X. campestris.*

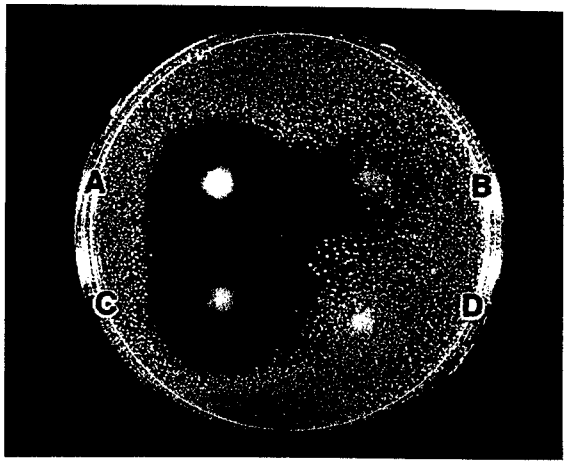

FIGURE 3. *(A) Formation of inhibition zones against* L. monocytogenes *Scott A on an iron-deficient medium (PAF) preinoculated with (A)* P. fluorescens *CY091A, (B)* E. carotovora *subsp.* carotovora *SR 319A, (C)* P. viridiflava *PJ-08-6A, or (D)* X. campestris *XC-5A.*

DISCUSSION

The data presented here show that *L. monocytogenes* is capable of growing on potato tuber slices to fairly high titers in the absence of native microflora naturally present on plants. The endogenous plant defense mechanisms cannot prevent the growth of this human pathogen, and the

TABLE 2. *Inhibition of* L. monocytogenes *on agar media preinoculated with four soft rot bacteria*[a]

Medium	Zone of inhibition[b] (mm)			
	P. fluorescens	*P. viridiflava*	*E. carotovora*	*X. campestris*
PAF	13.4	9.2	0.5	<0.1
PAF + $FeCl_3$ (1 mM)	<0.1	2.7	1.1	<0.1
Nutrient agar	<0.1	0.8	<0.1	<0.1
Potato dextrose agar	5.4	7.6	0.5	1.1

[a] *P. fluorescens* CY091A, *P. viridiflava* PJ-08-6A, *E. carotovora* subsp. *carotovora* SR319A, and *X. campestris* XC-5A.
[b] Values represent the average of three experiments (three replicates in each experiment).

nutrients released from the cut surface are sufficient to support the growth of *L. monocytogenes* on potato tuber slices to at least 4 to 5 log units. Earlier studies *(2, 3, 5, 25)* have shown that *L. monocytogenes* is capable of growing on several raw vegetables stored at controlled- or uncontrolled-atmosphere conditions. Because raw vegetables used in earlier studies *(2, 3, 5, 25)* were not surface-sanitized, it was not possible to determine whether native microflora present on the surface of vegetables influenced the growth of *L. monocytogenes.* Potato tubers used in this study were surface-sterilized, and slices thus prepared were free of microbial contamination. The ability of *L. monocytogenes* to grow on these surface-sanitized slices indicates that the presence of native microflora is not required for growth of this pathogen on potato slices.

Pectolytic bacteria are a major group of native microflora found on the surface of plants and are responsible for a large proportion of spoilage of fresh produce in storage or in transit *(14, 18, 19).* Previously, it was reported that *Salmonella* is more often associated with soft-rotted tissue than with healthy tissue *(26).* Carlin et al. *(6)* also showed a positive correlation between growth of *L. monocytogenes* and extent of spoilage on endive leaves after storage. In this study, we found that growth of *L. monocytogenes* on potato tuber slices was differentially affected by soft rot bacteria. Inhibition of *L. monocytogenes* was observed in potato tuber slices coinoculated with *P. fluorescens* or *P. viridiflava,* but very little or no inhibition was observed in slices coinoculated with *E. carotovora* or *X. campestris.* Furthermore, failure of *L. monocytogenes* to colonize the macerated tissue that was freshly induced by soft rot bacteria was possibly due to the preestablishment of high densities of soft-rot bacteria and the accumulation of high levels of antimicrobial compounds by fluorescent pseudomonads in macerated tissue. The possibility that soft-rotted tissue may serve as a source of growth of *Salmonella* or *Listeria,* as suggested in earlier studies *(6, 26),* is not clear and may be dependent on the age of macerated tissue and the bacterium used to induce maceration. In a separate study, Carlin et al. *(7)* also showed that resident strains of *P. fluorescens* isolated from endive inhibited the growth of *L. monocytogenes* on endive leaves.

Although the ability of *P. fluorescens* strains to inhibit growth of *L. monocytogenes* on endive leaves *(7)* and spinach *(1)* has been reported, the mechanism of inhibition has not been investigated. Several lines of evidence presented here indicate that the antagonism of *P. fluorescens* strains to *L. monocytogenes* possibly results from the production of a fluorescent siderophore by these pseudomonads. The inhibitory effect was observed only in iron-deficient media and correlated with the production of pyoverdin in culture media and potato tuber tissue extract. In addition, the *P. fluorescens gacA*$^-$ mutant deficient in production of pyoverdin lost the ability to inhibit growth of *L. monocytogenes* in potato tuber extract. It has been reported that some *P. fluorescens* strains isolated from soil *(13)* or poultry *(8)* can also suppress the growth of foodborne pathogens by producing metal-chelating siderophores. However, some *P. fluorescens* strains isolated from milk have been shown to enhance the growth of *L. monocytogenes* in milk *(9, 17),* possibly as a result of the production of proteases and lack of production of siderophores by these pseudomonads.

Chemical treatments and controlled-atmosphere packaging are the standard methods used to maintain the organoleptic and commercial quality of fresh and minimally processed fruits and vegetables. This practice reduces the number of native microflora, including the beneficial members, on the surface of produce, thereby creating a less competitive environment and a longer shelf life of fresh produce, which support the growth of human pathogens *(18).* To reduce the risk of pathogen growth, it is important not to recontaminate fresh or fresh-cut fruits and vegetables with pathogens after disinfection treatments. This study also raises the possibility of using native fluorescent pseudomonads that are naturally associated with plants and that do not cause soft rot to control the growth of *L. monocytogenes* on fresh-cut produce.

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