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Freeze-dried Spinach Powder Inhibits Growth of *Listeria* Species and Strains in Tryptic Soy Broth

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Abstract. **A study was undertaken to determine the inhibitory effect of freeze-dried spinach (*Spinacia oleracea* L.) powder and its native mesophilic aerobic microorganisms (MAM) on the growth of six strains, representing three species, of *Listeria* in tryptic soy broth (TSB). The cultures were incubated at 10 °C and growth was measured daily. Both spinach powder and MAM inhibited growth; maximum populations at 6 days were 8.8 Log_{10} colony-forming units (cfu) per mL^{-1} in control, 6.4 in spinach powder cultures, and 7.4 in MAM cultures. These results indicated that growth of various *Listeria* species/strains was affected by the bacteriostatic effect of spinach powder and competitive effect of native microorganisms.**

Within the genus *Listeria*, *L. monocytogenes* is the species involved in food poisoning. Most human listerioses are caused by the consumption of food from animal origin, mainly meat and dairy products (Eley, 1996). However, *L. monocytogenes* may also contaminate fresh raw vegetables (Farber et al., 1989; Heisick et al., 1989). Fresh-cut vegetables, such as coleslaw, lettuce, and prepacked salads, have been implicated in causing a few outbreaks of listeriosis (Schlech et al., 1983). This bacterium is of great concern to the fresh-cut industry since it is a psychrotroph that can grow on vegetables at temperatures <10 °C (Beuchat et al., 1990). The survival of *L. monocytogenes* has been studied on several fresh vegetables. Of particular interest is the inhibitory effect of raw, sliced, or shredded carrots on its growth (Beuchat and Brackett, 1990; Nguyen-the and Lund, 1991). Recently, Babic et al. (1997) reported that freeze-dried spinach powder restricted the growth of *L. monocytogenes* ATCC 19111 in tryptic soy broth (TSB), and that the native mesophilic aerobic microorganisms (MAM) of fresh-cut spinach played a role in the inhibitory effect. The objective of the present study was to determine if the inhibitory effect of freeze-dried spinach powder and its native MAM could be demonstrated on other species and strains of *Listeria*, including the nonpathogenic species *L. innocua* and *L. welshimeri*. The Gompertz function was used to characterize the growth of *Listeria* species and strains.

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Materials and Methods

Plant material. Fresh-cut spinach was obtained from a local supermarket. The leaves were washed twice with tap water, frozen in liquid nitrogen, freeze-dried, ground to a powder, and stored at –70 °C.

Microorganism used. L. monocytogenes Scott A, which was isolated from a patient affected in a Massachusetts outbreak that involved pasteurized milk (Fleming et al., 1985), and *L. monocytogenes* LCDC 81-861, which was isolated from coleslaw implicated in the 1981 listeriosis outbreak in Canada (Schlech et al., 1983), were supplied by L.R. Beuchat (Univ. of Georgia). *L. monocytogenes* ATCC 19111 isolated from meat, *L. monocytogenes* ATCC 7973, *L. innocua* ATCC 33090, and *L. welshimeri* ATCC 35897 were supplied by the Food Safety Inspection Service (U.S. Dept. of Agriculture, Beltsville, Md.). All species and strains were maintained on tryptic soy agar (TSA) (Difco Laboratories, Detroit) at 4 °C. An undefined culture of MAM was obtained by incubating 10 $mg \cdot mL^{-1}$ of spinach powder in TSB (Difco Laboratories) at 30 °C for 24 h. The culture was centrifuged at 2000 *g* for 20 min to recover only the spinach powder. An aliquot of the supernatant containing the cells was inoculated in fresh TSB and incubated at 30 °C for 24 h to obtain the culture of MAM.

To produce inocula, microorganisms were subcultured twice in TSB and incubated at 30 °C for 24 h.

Assays. Microbiological assays were conducted as described by Babic et al. (1997). Pure TSB or TSB containing either 10 $mg \cdot mL^{-1}$ spinach powder or an initial MAM population of $\approx 10^4$ $cfu \cdot mL^{-1}$, was inoculated with a 10^{-3} or a 10^{-5} dilution of the *Listeria* inocula in sterile NaCl solution (8.5 $g \cdot L^{-1}$) to obtain a final population of *Listeria* of $\approx 10^2$ (low inoculum) or 10^4 $cfu \cdot mL^{-1}$ (high inoculum). Inoculated broth at a pH of 7.0 was incubated at 10 °C for 6 d. The initial and final populations of viable microorganisms were determined by surface plate counts. Fifty µL of each sample was plated on the surface of an agar plate of the appropriate medium and incubated at 30 °C for 24 to 36 h. Colonies of *Listeria* were counted on Oxford medium used with Bacto Oxford antimicrobic supplement (Difco). Colonies of MAM were counted on TSA. Colonies of *Listeria* spp. were distinguished from colonies of other mesophilic aerobic microorganisms by colony morphology and excluded from the counts.

Statistical analysis. The microbial populations of *Listeria* and MAM were measured daily and the data were analyzed as a four-factor general linear repeated measures mixed model using the Statistical Software Procedure PROC MIXED (SAS Institute, 1996). *Listeria* strains, treatment (TSB with spinach, MAM, or no additive), and day were the fixed effects. Experiment was the random factor and means were compared using pair-wise contrasts.

The Gompertz function was used to determine if the growth characteristics of *Listeria* were affected by treatment, inoculum level, or species/strains using SAS Institute's Statistical Software PROC NLIN. The estimates for the growth parameters were analyzed as two-factor linear models using PROC MIXED where treatment and species/strains were the factors. The high and low inoculum experiments were analyzed separately.

The Gompertz function was defined as follows (Buchanan and Phillips, 1990):

$$L(t) = A + Ce^{-e[-B(t-M)]}$$

where L(t) = population of bacteria at time t (h) expressed in Log_{10} $cfu \cdot mL^{-1}$; A = asymptotic population of bacteria as time decreases indefinitely (i.e., initial population of bacteria) expressed in Log_{10} $cfu \cdot mL^{-1}$; C = asymptotic amount of growth, as time increases indefinitely, expressed in Log_{10} $cfu \cdot mL^{-1}$; M = time (h) at which the absolute growth rate is maximum; B = relative growth rate at M, expressed in Log_{10} $cfu \cdot mL^{-1} \cdot h^{-1}$.

Table 1. Analysis of variance of the growth (population) of *Listeria* species and strains as affected by growth media [tryptic soy broth (TSB) with spinach powder, mesophilic aerobic microorganisms, or without additives] over a 6-d period.

Source	df	F value	*P* value
Treatment	2	90.40	0.0001
Day	5	5792.30	0.0001
Listeria species/strains	5	17.19	0.0001
Treatment × day	10	98.74	0.0001
Treatment × species/strains	10	0.17	0.9980
Day × species/strains	25	9.38	0.0001
Treatment × day × species/strains	50	0.84	0.7547

Results and Discussion

Listeria population increased significantly with time (day) and the growth rate differed among the species/strains (Table 1). Growth was inhibited by the spinach powder and MAM when compared with growth in pure TSB (Table 2). The inhibition occurred on day 1 with spinach powder treatment and on day 3 with MAM treatment, which resulted in a significant interaction between treatment and time (Table 1). The growth of different species/strains was affected similarly by the treatments as indicated by lack of treatment × species/strains interaction. The growth of *L. innocua* and *L. welshimeri* was greater than that of the four strains of *L. monocytogenes* (Table 3), which resulted in the significant day × species/strains interaction (Table 1). The growth was not affected by the pH, which was 7.0 during incubation in all samples (Babic et al., 1997).

The analysis of variance of the Gompertz's functions showed that treatment and species/strains had different effects on growth parameters. Treatment had an effect only on the asymptotic amount of growth of all *Listeria* as time increased indefinitely (parameter C) and on the relative growth rate when the absolute growth rate was maximal (parameter B) under low or high inoculum (data not shown). Species/strains had an effect only on the asymptotic log count of *Listeria* under low or high inoculum as time decreased indefinitely (parameter A). Both the growth (C) and growth rate (B) of *Listeria* at low or high inoculum, in MAM or spinach powder cultures, were significantly different ($P < 0.05$) from that in the control TSB (Table 4). However, differences between MAM and spinach powder cultures were nonsignificant. The asymptotic log count of *Listeria* species/strains as time decreased indefinitely (A) differed slightly under low inoculum and significantly under high inoculum (Table 5).

The growth of the various species and/or strains of *Listeria* in TSB at 10 °C was restricted when freeze-dried spinach powder or an undefined population of MAM isolated from the same powder was added to the medium, and the restriction was similar among the species/strains. The growth of all *Listeria* spp. tested was also efficiently described by the Gompertz's function. Babic et al. (1997) reported that native microorganisms (MAM) of fresh-cut spinach restricted the growth of *L. monocytogenes* ATCC 19111. The present study demonstrates a bacteriostatic effect of MAM from spinach against the various species and strains of *Listeria* tested. *Listeria* strains from different sources, i.e., vegetable, animal, and human, were inhibited similarly by and grew similarly in TSB with spinach. In our earlier study, the growth of *L. monocytogenes* ATCC 19111 was restricted with increasing MAM population or with decreasing concentration of *Listeria* inoculum (Babic et al., 1997). Based on this, we postulated that the inhibitory effect of MAM on *L. monocytogenes* was the result of competition for nutrients; this probably explains inhibition of growth in this study. Beuchat and Brackett (1990) reported that the presence of carrot juice in tryptic phosphate broth reduced the maximum population of *L. monocytogenes*. Unidentified compounds may be responsible for the lethal effect of carrots and carrot juice (Nguyen-the and Lund, 1992). An inhibitory compound or physical structure in spinach might affect growth of *L. monocytogenes*, but this was not substantiated by Babic et al. (1997).

Table 2. Estimated populations (Log_{10} cfu·mL^{-1}) of *Listeria* in pure tryptic soy broth (TSB) or in TSB containing either spinach powder or a population of mesophilic aerobic microorganisms (MAM) during 6 d of incubation at 10 °C. Means for all species/strains.

	Culture			
Day	Pure	+Spinach powder	+MAM	Day means
0	3.41 a[zy]	3.39 a[x]	3.41 a[y]	3.40[y]
1	4.44 a	4.16 b	4.41 a	4.34
2	5.55 a	5.05 b	5.36 a	5.32
3	6.68 a	5.82 b	6.54 c	6.35
4	7.81 a	6.24 b	7.01 c	7.02
6	8.86 a	6.46 b	7.41 c	7.58
Treatment means	6.12	5.19	5.72	

[z]Mean separation within rows (letters) by pair-wise contrast at $P < 0.05$
[y]All means within column different from one another by pair-wise contrast, $P < 0.01$.
[x]All means within column different from one another by pair-wise contrast, $P < 0.05$.

Table 3. Estimated populations (Log_{10} cfu·mL^{-1}) of *Listeria* species/strains in tryptic soy broth during 6 d of incubation at 10 °C.

	Listeria monocytogenes					
Day	19111	81-861	Scott A	7973	*L. innocua*	*L. welshimeri*
0	3.48 b[zy]	3.18 c[y]	3.17 c[y]	3.33 bc[y]	3.77 a[y]	3.49 b[x]
1	4.28 bc	4.09 c	4.19 c	4.22 c	4.76 a	4.47 b
2	5.25 c	5.19 cd	5.33 c	5.07 d	5.82 a	5.62 b
3	6.18 d	6.31 cd	6.38 bc	5.79 e	6.86 a	6.56 b
4	6.74 d	7.01 c	7.07 bc	6.47 e	7.58 a	7.25 b
6	7.33 cd	7.51 bc	7.67 bc	7.05 d	8.11 a	7.79 ab
Means	5.55 o[w]	5.55 o	5.64 o	5.32 p	6.15 m	5.86 n

[z]Mean separation within rows (letters) by pair-wise contrast, $P < 0.05$.
[y]All means within column different from one another by pair-wise contrast, $P < 0.01$.
[x]All means within column different from one another by pair-wise contrast, $P < 0.05$.
[w]Mean separation among species/strains by pair-wise contrast, $P < 0.05$.

Table 4. Asymptotic amount of growth (Log_{10} cfu·mL^{-1}) of *Listeria* as time increases indefinitely (Gompertz's parameter C), and relative growth rate (Log_{10} cfu·mL^{-1}·h^{-1}) when the absolute growth rate is maximal (Gompertz's parameter B) under low or high inoculum (2.0 or 4.0 Log_{10} cfu·mL^{-1}, respectively) in pure tryptic soy broth (TSB), TSB containing mesophilic aerobic microorganisms (MAM), or TSB containing spinach powder. Means for all species/strains.

	Low inoculum		High inoculum	
Treatment	C	B	C	B
TSB	9.31 a[z]	0.017 a	5.36 a	0.027 a
MAM	4.91 b	0.031 b	3.13 b	0.038 b
Spinach	5.22 b	0.029 b	4.10 c	0.037 b

[z]Mean separation within columns by pair-wise contrast, $P < 0.05$.

Table 5. Mean asymptotic log count (Log_{10} cfu·mL^{-1}) of various *Listeria* species/strains over all treatments as time decreases indefinitely (Gompertz's parameter A) under low or high inoculum (2.0 or 4.0 Log_{10} cfu·mL^{-1}, respectively).

Species/strains	Low inoculum	High inoculum
L. m. ATCC 19111	1.98 ab[z]	4.18 c
L. m. LCDC 81-861	---	4.77 a
L. m. Scott A	1.29 b	3.84 d
L. m. ATCC 7973	1.09 ab	3.82 d
L. innocua	2.40 a	4.52 b
L. welshimeri	1.57 b	4.41 b

[z]Mean separation within columns by pair-wise contrast, $P < 0.05$.

Literature Cited

Babic, I., A.E. Watada, and J.G. Buta. 1997. Growth of *Listeria monocytogenes* restricted by native microorganisms and other properties of fresh-cut spinach. J. Food Protection 60:912–917.

Beuchat, L.R., M.E. Berrang, and R.E. Brackett. 1990. Presence and public health implications of *Listeria monocytogenes* on vegetables, p. 175–181. In: A.J. Miller, J.L. Smith, and G.A. Somkuti (eds.). Foodborne listeriosis. Elsevier, Amsterdam.

Beuchat, L.R. and R.E. Brackett. 1990. Inhibitory effect of raw carrots on *Listeria monocytogenes*. Appl. Environ. Microbiol. 56:1734–1742.

Buchanan, R.L. and J.G. Phillips. 1990. Response surface model for predicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. J. Food Protection 53:370–376.

Eley, A.R. 1996. Microbial food poisoning. 2nd ed. Chapman and Hall, London.

Farber, J.M., G.W. Sanders, and M.A. Johnston. 1989. A survey of various food for the pres-

ence of *Listeria* species. J. Food Protection 52:456–458.

Fleming, D.W., S.L. Cochi, K.L. MacDonald, J. Brondum, P.S. Hayes, B.D. Plikatis, M.B. Holmes, A. Audurier, C.V. Broome, and A.L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. New Eng. J. Med. 312:404–407.

Heisick, J.E., D.E. Wagner, M.L. Nierman, and J.T. Peeler. 1989. *Listeria* spp. found on fresh market produce. Appl. Environ. Microbiol. 55:1925–1927.

Nguyen-the, C. and B.M. Lund. 1991. The lethal effect of carrot on *Listeria* species. J. Appl. Bacteriol. 70:479–488.

Nguyen-the, C. and B.M. Lund. 1992. An investigation of the antibacterial effect of carrot on *Listeria monocytogenes*. J. Appl. Bacteriol. 73:23–30.

SAS Institute. SAS/STAT software. 1996. Changes and enhancements through release 6.11. SAS Inst., Cary, N.C.

Schlech, W.F., P.M. Lavigne, R.A. Bortolussi, A.C. Allen, E.V. Haldene, A.J. Wort, A.W. HighTower, S.E. Johnson, S.H. King, E.S. Nicholls, and C.V. Broome. 1983. Epidemic listeriosis—Evidence for transmission by food. New Eng. J. Med. 308:203–206.