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Effects of pH on Distribution of *Listeria* Ribotypes in Corn, Hay, and Grass Silage

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***Listeria* spp. isolated from 13 of 129 (10%) corn silage samples, 21 of 76 (28%) hay silage samples, and 3 of 5 (60%) grass silage samples during a previous Vermont survey were subjected to automated ribotype (RT) analysis. The 13 positive corn silage samples contained 3 *Listeria monocytogenes* isolates (three RTs, including one known clinical RT) and 10 *L. innocua* isolates (four RTs). Similarly, 2 *L. monocytogenes* isolates (two RTs) and 19 *L. innocua* isolates (three RTs) were identified in the 21 positive hay silage samples. The three positive grass silage samples contained two *L. innocua* isolates (two RTs) and one isolate of *L. welshimeri*. One hundred seven of 129 (83%) high-quality (pH < 4.0) corn silage samples accounted for 8 of 13 *Listeria* isolates from corn silage, including isolates belonging to one *L. monocytogenes* clinical RT. In contrast, low-quality hay silage (70 of 76 [92%] samples having a pH of ≥4.0) harbored 20 of 21 isolates, including isolates belonging to two nonclinical *L. monocytogenes* RTs. Poor-quality silage is readily discernible by appearance; however, these findings raise new concerns regarding the safety of high-quality (pH <4.0) corn silage, which can contain *Listeria* spp., including *L. monocytogenes* strains belonging to RTs of clinical importance in cases of food-borne listeriosis.**

An association between silage consumption and listeriosis in ruminants was first recognized in 1922, when investigators warned of a disease resembling listeriosis known in Iceland as "votheysveiki," or silage sickness (9). This relationship between listeriosis and the feeding of silage to dairy cattle (4, 5, 20), sheep (4, 7, 10, 14, 20), and goats (5, 20) has been well documented, with most cases resulting from the consumption of low-quality, improperly fermented silage having a pH of >4.0. Reports of bovine listeriosis from silage feeding and of subsequent asymptomatic shedding of *Listeria monocytogenes* in milk (6) are of obvious concern to the dairy industry.

Listeria spp., including *L. monocytogenes*, are most commonly recovered from improperly fermented silage (8, 20). However, detailed information concerning the incidence of *Listeria* strains in silage is still largely lacking, since most surveys to date were conducted well before strain-specific typing became feasible. By analyzing hemolysin production and virulence, and through the use of serology and phage typing, Fensterbank et al. (5) found that 12 *L. monocytogenes* and 27 *L. innocua* isolates from silage belonged to three and two distinct types, respectively. In the few other existing studies (3, 15, 16, 23), various combinations of serotyping, phage typing, multilocus enzyme electrophoresis, and pyrolysis mass spectroscopy were used to link the consumption of *Listeria*-contaminated silage to scattered cases of bovine and ovine listeriosis. Consequently, *Listeria* isolates obtained from a previously reported large-scale survey of Vermont silage were reevaluated (i) to determine the incidence of strain-specific ribotypes (RTs) of *L. monocytogenes* and other *Listeria* spp. in corn, hay, and grass silage, with particular emphasis given to RTs of recognized clinical importance; and (ii) to assess the correlation between the incidence of *Listeria* RTs and silage pH.

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***Listeria* strains.** A total of 37 *Listeria* strains isolated from 13 of 129 corn silage samples, 21 of 76 hay silage samples, and 3 of 5 grass silage samples during a 1989 survey of Vermont silage (19) were ribotyped (Table 1). These strains, which were preserved at −70°C in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) containing 10% glycerol (Sigma Chemical Co., St. Louis, Mo.), were removed from frozen storage and subcultured twice on brain heart infusion agar (Difco) (18 to 24 h at 37°C) before being ribotyped.

RT analysis. All 37 *Listeria* strains were ribotyped by the automated Riboprinter microbial characterization system, alpha version, developed by E. I. du Pont de Nemours & Co., Inc. (Wilmington, Del.) (2, 11). This six-stage, largely automated process used to identify selected *Listeria* strains to the species level and further characterize them is based on the simultaneous separation and transfer of *Eco*RI DNA restriction fragments, followed by hybridization with a chemiluminescently labeled DNA probe from *Escherichia coli* encoding rRNA.

Analysis of pH. The pH data for all silage samples were previously obtained by Perry and Donnelly (19), who used a pH meter (Orion Research, Cambridge, Mass.) equipped with a standard combination electrode.

Overall diversity of *Listeria* RTs. The *Listeria* isolates ribotyped in this study were obtained from a 1989 survey of Vermont silage conducted by Perry and Donnelly (19) in which 13 of 129 (10%) corn silage samples, 21 of 76 (28%) hay silage samples, and 3 of 5 (60%) grass silage samples were positive for listeriae (Table 1). These isolates belonged to a diverse group of RTs. A total of 11 distinct *Listeria* RTs comprising 5 of 47 *L. monocytogenes* strains, 5 of 29 *L. innocua* strains and 1 of 8 *L. welshimeri* strains in the Riboprinter microbial characterization system data base (alpha version) were identified. Corn silage contained the highest number of *Listeria* RTs (seven RTs), followed by hay silage (five RTs) and grass silage (three RTs). One of three *L. monocytogenes* isolates from corn silage belonged to a known clinical RT (19193) previously

TABLE 1. Incidence of *Listeria* RTs in corn, hay, and grass silage

Listeria sp.	RT	No. (%) of isolates in type of silage			Total (n = 210)
		Corn (n = 129)	Hay (n = 76)	Grass (n = 5)	
L. monocytogenes	19092	1 (0.8)			1 (0.5)
	19193[a]	1 (0.8)			1 (0.5)
	54183	1 (0.8)			1 (0.5)
	19071		1 (0.8)		1 (0.5)
	19075		1 (0.8)		1 (0.5)
L. innocua	19073	1 (0.8)	8 (10.5)		9 (4.3)
	19094	7 (5.4)	10 (13.2)	1 (20)	18 (8.6)
	19166	1 (0.8)	1 (1.3)		2 (1.0)
	19181			1 (20)	1 (0.5)
	19196	1 (0.8)			1 (0.5)
L. welshimeri	19214			1 (20)	1 (0.5)
Total		13 (10)	21 (28)	3 (60)	37 (18)

[a] Known clinical RT associated with food-borne listeriosis.

associated with human cases of listeriosis and linked to the consumption of cream in England (17, 21). Three *L. innocua* RTs (19073, 19166, and 19094) were common to corn and hay silage, with RT 19094 dominating and also appearing in grass silage. *L. welshimeri* (RT 19214) was confined to a single sample of grass silage.

***Listeria* RTs and silage pH.** Three different *Listeria* RTs were identified in 2 of 17, 1 of 3, and 2 of 2 corn silage samples having pH values of 4.00 to 4.99, 5.00 to 5.99, and ≥6.00, respectively (Table 2). Although 107 of the 129 (83%) corn silage samples examined previously were of excellent quality (pH 3.75 to 3.97), the same samples accounted for 8 of 13 (62%) *Listeria* isolates, including the only clinical RT of *L. monocytogenes*. Similar findings attesting to the acid tolerance of listeriae were also reported by Fensterbank et al. (5), who identified *Listeria* spp., including *L. monocytogenes*, in 11 of 31 high-quality silage samples with pHs of 3.6 to 4.0. Given the abundance of high-quality corn silage currently available in Vermont and elsewhere, these findings raise new concerns regarding the role of supposedly high-quality, properly fermented silage in the spread of listeriosis. Hay, which contains lower levels of fermentable carbohydrates than corn, produced silage of far lower quality, with 70 of 76 samples having a pH of ≥4.00 (Table 3). These low-grade silage samples yielded 20 of the 21 *Listeria* isolates, including strains belonging to two RTs of *L. monocytogenes* and three RTs of *L. innocua*. Only 1 of the 21 *Listeria*-positive samples was of high quality (pH <4.00). The three *Listeria*-positive grass silage samples were all of poor quality, ranging in pH from 5.78 to 5.89.

TABLE 2. Distribution of *Listeria* RTs in corn silage according to pH

Listeria sp.	RT	No. (%) of isolates at pH			
		<4.00 (n = 107)	4.00–4.99 (n = 17)	5.00–5.99 (n = 3)	>6.00 (n = 2)
L. monocytogenes	19092	1 (0.9)			
	19193[a]	1 (0.9)			
	54183		1 (5.9)		
L. innocua	19073	1 (0.9)			
	19094	4 (3.7)	1 (5.9)	1 (33)	1 (50)
	19166	1 (0.9)			
	19196				1 (50)
Total		8 (7)	2 (12)	1 (33)	2 (100)

[a] Known clinical RT associated with food-borne listeriosis.

TABLE 3. Distribution of *Listeria* RTs in hay silage according to pH

Listeria sp.	RT	No. (%) of isolates at pH			
		<4.00 (n = 6)	4.00–4.99 (n = 52)	5.00–5.99 (n = 13)	>6.00 (n = 5)
L. monocytogenes	19071			1 (7)	
	19075			1 (7)	
L. innocua	19073		3 (5.8)	1 (7)	4 (80)
	19094	1 (17)	6 (11.5)	6 (11.5)	1 (20)
	19166			1 (7)	
Total		1 (17)	9 (17)	10 (77)	5 (100)

The high prevalence of pathogenic listeriae in improperly fermented (pH ≥ 4.0), overtly spoiled hay silage again legitimizes concerns regarding the spread of animal listeriosis. While prescreening for such poor-quality silage as part of an on-farm hazard analysis critical control point (HACCP) program should sharply decrease the incidence of listeriosis in livestock, minimizing the use of properly fermented (pH < 4.0), high-quality corn silage containing *L. monocytogenes* RTs of recognized clinical importance in human food-borne illness presents a far greater challenge. Since silage is one presumed source of *L. monocytogenes* strains that contaminate processing facilities (1), several recent reports indicating the increased thermal (12) and chemical (13) resistance as well as the increased virulence (18) of acid-adapted cells should also be of concern to the dairy industry. Given the well-documented relationship between the ingestion of *Listeria*-contaminated silage, mastitis in dairy cattle, and subsequent asymptomatic shedding of listeriae in milk destined for human consumption (22), it is imperative that HACCP programs for control of *Listeria* and other food-borne pathogens begin first and foremost at the farm level.

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