Survival of *Listeria monocytogenes* in Wilted and Additive-Treated Grass Silage

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Pauly TM, Tham WA: Survival of *Listeria monocytogenes* in wilted and additive-treated grass silage. Acta vet. scand. 2003, 44, 73-86. – Grass was field-dried to 3 different dry matter (DM) levels (200, 430 and 540 g/kg) and inoculated with 10^6-10^7 cfu/g of a *Listeria monocytogenes* strain sharing a phagovar occasionally involved in foodborne outbreaks of listeriosis. Formic acid (3 ml/kg) or lactic acid bacteria ($8 \cdot 10^5$/g) with cellulolytic enzymes were applied only to forages with low and intermediate DM levels. Forages were ensiled in laboratory silos (1700 ml) and were stored at 25 °C for 30 or 90 days. After 90 days of storage, *L. monocytogenes* could not be detected in any silo, except one with the high dry matter grass without additive. After 30 days of storage, between 10^2 and 10^6 cfu *L. monocytogenes*/g silage were isolated from the untreated silages. Increasing the DM content from 200 to 540 g/kg did not reduce listeria counts possibly because of the lower production of fermentation acids (higher pH). In silages treated with additives, counts of *L. monocytogenes* were always lower than in silages without additive. In wet silages (DM 200 g/kg) both additives were effective, but in the wilted silages (DM 430 g/kg) only the bacterial additive reduced listeria counts below detection level. Listeria counts were highly correlated to silage pH (r = 0.92), the concentration of lactic acid (r = -0.80) and the pooled amount of undissociated acids (r = -0.83).

additive; formic acid; lactic acid bacteria; wilting; dry matter; water activity; pH.

Introduction

Listeria monocytogenes is the causative organism of the disease listeriosis which affects both man and a wide range of animals with manifestations such as septicaemia and/or affection of the central nervous system. Immunosuppressive conditions are predisposing; e.g., pregnancy leading to infection of the foetus and thus to abortion. Among farm animals, sheep appear to be particularly susceptible to listeriosis. The organism is spread world-wide in nature (decaying herbage, soil, faeces, sewage) and occurs usually in low numbers (*Fenlon* 1988, *Woolford* 1990). While listeriosis in animals has been associated with silage-feeding since 1960 (*Seeliger* 1961, *Gray & Killinger* 1966), it was not until the 1980s that *L. monocytogenes* became recognised as an important pathogen in the food chain, particularly in cold-stored, unfrozen food.

Husu (1990) examined the occurrence of *Listeria* spp. in 68 grass and 225 grass silage samples which were collected from 80 Finnish dairy farms. *Listeria innocua* or *L. monocytogenes* were isolated from 65% of the grass samples and 23% of the silage samples. *L. monocytogenes* was confirmed at least at one occasion in the silage of 34% of all farms. This investigation makes clear that at least in Scandinavia listeria are probably quite common in fresh forage and that ensiling *per se* will not guarantee a

listeria-free feed. Because there is no practical way to make fresh forage listeria-free, the best way to avoid proliferation in silage is to direct the ensiling process in such a way that listeria are unlikely to survive the storage period of the silage.

With an imperfect aerobic and a facultative anaerobic metabolism *L. monocytogenes* is stimulated by micro-aerophilic conditions as when air leaks into a silo (*Fenlon* 1986a). Farm silos or big bales sealed with polyethylene film cannot be considered completely gas-tight (*Woolford* 1990). The permeability of low density polyethylene film to oxygen and carbon dioxide increases exponentially as temperature rises though the permeation rate at any given temperature is always higher for carbon dioxide than for oxygen (*Möller et al.* 1999). However, the air leakage between film layers (big bales) or between plastic sheets and silo wall (bunker silos) might become much larger in badly sealed silos or bales than the actual permeation through the plastic film. Acidic conditions inhibit the growth of *L. monocytogenes,* but there is no consensus on the precise pH-level in silage at which the organism will cease to grow (*Irvin* (1968): no growth below pH 5.5; *Fenlon* (1988): slow growth at pH 4.5). This is not surprising because there are usually more environmental factors than just pH that affect the growth of listeria and other competing organisms. When freshly cut forage is dried in the field, the dry matter (DM) content will increase and the water activity[1] (a_w) decrease. The decreasing water activity reduces the activity of all viable bacteria, including lactic acid bacteria

(LAB), the main producers of acids in silage. Field drying or wilting will therefore lead to a lower production of fatty acids and thus a higher pH in the silage (*McDonald et al.* 1991). Regarding the growth of *L. monocytogenes* in wilted silage, it is not clear whether the inhibitory effect of the reduced water activity is compensated by the stimulating effect of the higher pH in the resulting silage. The objective of the present study was therefore to investigate how environmental factors such as dry matter, water activity, acidification (pH) and fermentation products (fatty acids) influenced the survival of *L. monocytogenes* in grass silage.

Materials and methods
Experimental plan
An ensiling experiment was conducted in small laboratory silos with ryegrass *(Lolium perenne)* in the stage of early ear emergence. The grass was cut in early August with a mower-conditioner and was wilted (field-dried) to 3 different DM levels (215, 453, 545 g/kg). After wilting, the grass was chopped in a stationary chopper head to approx. 5 cm length and spread indoors in an approx. 10 cm thick layer on a new plastic sheet. Forage for an uncontaminated control treatment was taken aside. First the *L. monocytogenes* suspension was applied to the forage equivalent to approx. 10^6-10^7 cfu/g grass (Table 2). About half of the contents were sprayed on the forage with a spray bottle before blending. Then the rest was added and the forage was remixed. Thereafter, silage additives (formic acid or lactic acid bacteria with cellulolytic enzymes) were applied in the same way to the moist and wilted grass fraction. No silage additives were applied to the high DM herbage (545 g/kg).

The added *L. monocytogenes* strain (SLU 376), originally isolated from farm silage, belongs to a certain serovar (4b) and phagovar (2389: 2425:3274:2671:47:108:340) associated with

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[1] Water activity is the ratio of the vapour pressure over the sample to that over pure water at a given temperature. Water activity of a feed sample correlates better to microbial growh than the water content of the sample (*Rödel* 1993).

food-borne outbreaks world-wide (*Tham et al.* 1994).

The 3 additive treatments were:
a) No additive (0)
b) Formic acid (FA), 3 ml formic acid (85% w/w) + 7 ml tap water per kg grass
c) Lactic acid bacteria (LAB), 10 ml/kg grass of the bacterial silage additive Siloferm Plus (Medipharm AB, S-260 23 Kågeröd, Sweden) resulting in 8×10^5 *Lactobacillus plantarum* per g forage plus cellulolytic enzymes (1.3 Econase cellulase units (ECU[2]) /g forage, Alko Ltd., FIN-05200 Rajamäki, Finland).

The grass was ensiled in 1700 ml-glass jars (diameter 11 cm, height 18 cm) in 3 replications per treatment, DM level and storage time. Jars were filled with 800, 650 and 600 g fresh matter depending on DM level (215, 453 and 545 g/kg, respectively). The jars were stored for 30 or 90 days in a temperature controlled room at $25 \pm 2°C$. (Table 1).

In order to create storage systems with two different levels of oxygen supply, either a water-filled water lock or an open capillary tube (inner diameter 0.4 mm, length 15 mm) were mounted on each silo lid. Untreated silos were sealed with both methods but additive-treated silos only with capillary tubes (Table 1). In water locks, only pressure differences larger than 40-50 mm water column (4-5 hPa) are released whereas a small but continuous gas exchange is made possible with capillary tubes. These 2 treatments were introduced because *L. monocytogenes* is said to thrive under micro-aerophillic conditions and is stimulated when small

amounts of air leak into the silo (*Fenlon* 1986a). A leaking storage system might resemble conditions in many farm silos.

Sampling and analyses

Forage samples and analyses. One grass sample was collected from each of the 3 DM levels. DM concentration was determined in 2 steps. The first drying was done in a ventilated oven at 65°C for 18 h. After grinding in a hammer mill (1 mm sieve), the second drying was done at 103°C for 3 h to evaporate remaining water. Nitrogen content was determined in a Kjeltec Auto 1030 Analyser according to the Kjeldahl method (*NMKL* 1976). Crude protein was calculated as N / 0.16. Water soluble carbohydrates (for simplicity here called "sugars") were extracted with boiling water and the extract was hydrolysed with hot 0.074 M sulphuric acid followed by an enzymatic determination of glucose and fructose (*Larsson & Bengtsson* 1983).

Silage samples and analyses. Samples for determinations of *L. monocytogenes* and water activity (approx. 100 g) were collected after storage periods of 30 and 90 days. The sample consisted of silage from the top of the jar down to a depth of approx. 6 cm. *L. monocytogenes* was isolated by both qualitative and quantitative procedures (detection level 100 cfu/g) (*Loncarevic et al.* 1996). Counts of *L. monocytogenes* were determined from ten-fold serial dilutions of samples in peptone water followed by surface-plating onto Listeria Selective Agar (Oxoid CM856 & SR140) and aerobic incubation for 48 h at 37°C. The remaining contents of each jar were emptied, mixed and divided into 3 equal subsamples for determination of DM concentration, pH, and fermentation products (ammonium nitrogen, organic acids, 2.3-butanediol). DM contents were determined as in grass samples, however, val-

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[2] Enzyme activity determined as ECU on hydroxylethyl cellulose substrate at 50°C, pH 4.8 and 10 min. incubation time. 1 ECU is defined as the activity producing 1 nmol glucose per second.

Table 1. Experimental plan showing number of silos per treatment (N=78). Formic acid (85%): 3 ml per kg grass; Lactic acid bacteria: 8 x 10^8 per kg grass.

Additive	L.m.* contami- nated	Storage period (days)	200 gDM/kg		430 gDM/kg		540 gDM/kg	
			Water lock	Capillary tube	Water lock	Capillary tube	Water lock	Capillary tube
No		90	3	3	3	3	3	3
additive	X	30	3	3	3	3	3	3
(0)	X	90	3	3	3	3	3	3
Formic acid	X	30		3		3		
(FA)	X	90		3		3		
Lactic acid	X	30		3		3		
bacteria (LAB)	X	90		3		3		

*: L.m. = *Listeria monocytogenes.*

ues were corrected for the loss of volatiles as described by *Rammer* (1996). Water activity was determined at 25 °C in a Humidat-TH2 (Novasina, Defensor AG, Pfäffikon, Switzerland) and pH in the silage juice with a pH-meter (model 654, Metrohm AG, Herisau, Switzerland). In high DM samples demineralised water was added in proportion 1:1 (w/w) before the silage juice was extracted. Ammonia-nitrogen was determined by direct distillation in a Kjeltec Auto-Analyzer 1030 (Tecator AB, Sollentuna, Sweden). Silage juice was analysed by HPLC (*Andersson & Hedlund* 1983) for determination of lactic, acetic, butyric and formic acid. The concentration of undissociated acid (C_{undis}; g/kgDM) was calculated from lactic, acetic or formic acid using the equation

$$C_{undis} = C / (1+10^{(pH - pKa)})$$

with C being the concentration of acid in the silage (g/kgDM), pH the silage pH, and pK_a the acid-specific pH at which one half of the acid is dissociated and the other undissociated.

Listeria counts were plotted against different silage constituents, and single and multiple regressions were made with the purpose to detect the most relevant parameters that affected liste-

ria survival. The pooled amount of lactic, acetic and formic acid was called "acids" and the pooled amount of undissociated lactic, acetic and formic acid was called "undissociated acids".

Statistical analyses

From the silage analyses treatment means and least significant differences (LSD) among treatments were calculated for different silage parameters. Microbiological counts were transformed to logarithmic values as suggested by *Niemelä* (1983). LSD values indicate the difference between treatment means for statistical significance at the 5% probability level (p=0.05). All differences between treatment means mentioned in the text below were statistically significant (p<0.05) if not stated otherwise.

Silage parameters were grouped into 2 data sets to facilitate pre-planned comparisons. In the first data set, only silos without additives were selected. Among contaminated silos ($n_1 = 36$), listeria growth was studied in relation to DM level (200, 430, 540 g/kg), length of storage period (30 vs. 90 days) or sealing method (water lock vs. capillary tube) by analysis of variance. Sealing method was, however, not significant

Table 2. Composition of fresh forages after contamination with *L. monocytogenes*.

DM g/kg	Water activity (25°C)	Crude protein g/kgDM	WSC* g/kg	*L. monocytogenes* cfu / g	log cfu / g
215	0.99	155	31	9×10^6	6.95
453	0.98	157	65	3×10^6	6.48
545	0.95	156	83	2×10^6	6.30

*: WSC = water soluble carbohydrates.

($R^2 = 0.0002$) and was omitted from the model. This led to a two-factorial design (3 DM levels, 2 storage periods) with 6 silo replications per treatment.

In the second data set, only listeria contaminated silages sealed with capillary tubes were selected (see Table 1). Here the influence of the 2 additives (formic acid and lactic acid bacteria) on listeria growth was compared with untreated control-silages in a two-factorial design (3 additive treatments, 2 storage periods) with 3 silo replications per treatment. This analysis was executed separately for the 200 and 430 DM level ($n_2 = 18$).

Finally, listeria counts were correlated to different silage constituents and regressions with one or two dependent variables were made with the objective to explain listeria survival in silages. Only data from contaminated silages sampled after 30 days were used (n = 30), because uncontaminated silages and silages sampled after 90 days were practically void of listeria. All variables in the presented models were significantly different from zero (p<0.05).

Results and discussion
Fresh forage
A concentration of water soluble carbohydrates (WSC) of at least 15 to 25 g/kg fresh matter is considered sufficient for an unrestricted lactic acid fermentation (*Pettersson* 1988, *Pahlow* 1990). The composition of the grass crop, presented in Table 2, shows that the WSC content was high at all 3 DM levels. Counts of *L. monocytogenes* in the 3 forage batches varied only slightly from 2×10^6 to 9×10^6 cfu/g. When the DM content increased from 215 to 545 g/kg, water activity was reduced from 0.99 to 0.95.

Silages stored for 90 days
After 90 days of storage, no culturable listeria were detected in any of the uncontaminated silages (data not shown) and among contami-

Table 3. Composition of contaminated silages without additives. Values are means of 6 silos (silos with water locks and capillary tubes were merged).

Silage analyses	200 gDM/kg 30 days	90 days	430 gDM/kg 30 days	90 days	540 gDM/kg 30 days	90 days	LSD*
DM content (g/kg)	206	201	425	423	536	538	8.3
Water activity (a_w)	0.99	0.97	0.96	0.96	0.95	0.95	0.018
pH	4.90	4.65	5.80	5.53	5.93	5.88	0.057
L. monocytogenes (log cfu/g)	2.3	n d[+]	7.9	n d	6.3	<2.0*	0.94
Lactic acid (g/kgDM)	70	86	32	44	10	14	1.9
Acetic acid (g/kgDM)	8	11	4	5	4	4	0.5
Butyric acid (g/kgDM)	17	21	0	0	0	0	0.5
Ammonia-N (g/kg N)	99	110	58	82	32	43	2.6

* *L. monocytogenes* was detected only in 1 (<log2.0/g) out of 6 silos.
[+] nd = *L. monocytogenes* not detected; *LSD = least significant difference at p = 0.05.

Table 4. Composition of contaminated silages with and without additives. Values are means from 3 silos.

Silage analyses	No additive		Formic acid		Lactic acid bacteria		LSD*
	30 days	90 days	30 days	90 days	30 days	90 days	
Low DM level (200 gDM/kg)							
DM content (g/kg)	205	202	206	203	208	201	3.1
Water activity (a_w)	0.99	0.99	0.98	0.97	0.99	0.95	0.016
pH	4.90	4.70	4.27	4.20	3.90	3.93	0.139
L. monocytogenes (log cfu/g)	2.2	nd	nd	nd	nd	nd	1.60
Lactic acid (g/kgDM)	71	83	79	88	130	133	11.1
Acetic acid (g/kgDM)	8	12	11	22	10	9	1.8
Formic acid (g/kgDM)	0	0	3	4	0	0	-
Butyric acid (g/kgDM)	17	21	0	3	0	0	2.3
Ammonia-N (g/kg N)	100	114	74	89	69	72	9.5
Medium DM level (430 gDM/kg)							
DM content (g/kg)	424	422	441	420	433	416	10.4
Water activity (a_w)	0.96	0.96	0.98	0.97	0.95	0.95	0.008
pH	5.80	5.53	5.53	5.43	4.10	4.30	0.073
L. monocytogenes (log cfu/g)	7.9	nd	4.2	nd	nd	nd	0.44
Lactic acid (g/kgDM)	31	43	10	14	86	95	5.8
Acetic acid (g/kgDM)	4	5	2	3	5	6	0.3
Formic acid (g/kgDM)	0	0	5	4	0	0	-
Butyric acid (g/kgDM)	0	0	0	0	0	0	-
Ammonia-N (g/kg N)	58	81	28	45	47	46	1.8

[+] nd = L. monocytogenes not detected; *LSD = least significant difference at p = 0.05.

nated silages listeria were detected only in one out of 30 silos despite a high contamination dose and relatively high pH values in the silages (Tables 3 and 4). In this particular silo (DM 514 g/kg, 0.975 a_w, pH 5.9), counts of *L. monocytogenes* were just about detectable ($<10^2$/g). In the other 5 silo replications, no culturable listeria were found. However, these 5 silages had slightly higher DM contents (mean 542 g/kg) and lower water activity values (mean 0.948) (differences not significant), which might have been sufficient to prevent listeria survival past day 90. This indicated that even in silages with a pH up to approx. 5.8, high initial counts of listeria might be eliminated, if the storage time is at least 90 days.

A possible explanation for why high counts of listeria could be found in some silages after 30 days but had disappeared after 90 days might be that slightly more acid was formed after day 30. A long storage period might decrease counts if conditions are unfavourable for growth (lower pH) and if listeria are exposed to competitive interaction from other silage microorganisms.

Silages stored for 30 days without additive
Because we were not able to record the actual ingress of air or oxygen into the silos, we can only speculate about the cause for the lacking difference between silos sealed with water locks and capillary tubes. Moulds do generally require oxygen for growth. Mould growth is

therefore closely related to the quantity of air which leaks into the silo (*Woolford* 1990). The absence of any mould growth on silages indicated that the ingress of air through the capillary tubes must have been very limited. It is, however, possible that droplets of condensed water might have clogged the capillary tubes at times.

Wet, unwilted silages without additive fermented badly. High concentrations of butyric acid and ammonia indicated growth of *Clostridium* spp. Levels of lactic and acetic acid were low in all silages without additives and, consequently, pH values were above critical levels. Well-fermented silages are expected to have pH values not higher than 4.4, 4.8 and 5.1 at DM levels of 200, 430 and 540 g/kg, respectively (*Weissbach* 1996). As the DM content increased, silage quality improved (no clostridial activity) but pH values were still high.

Listeria counts varied widely among DM contents ($<10^2$ to 10^8 cfu/g). In the wettest silages (DM 200 g/kg), counts were reduced to a few hundred per gram, probably because the amounts of organic acids were higher and pH values lower than in silages with higher DM contents. Listeria counts increased in the drier silage (DM 430 g/kg) up to 100 million cfu/g and remained on the initial level at the highest DM level (Table 3). The increase in DM content from approx. 430 to 540 g/kg resulted in only a marginal decrease in water activity (not significant) and a slight increase in pH which could not explain the lower listeria counts in silages with the highest DM.

These results indicate that the increase in DM content to about 540 g/kg, equivalent to a reduction of water activity to 0.95, was not an efficient measure to eliminate listeria in silage with pH as high as 5.9.

Silages stored for 30 days treated with additives
The application of formic acid stopped listeria

growth efficiently at DM 200 g/kg, but not at DM 430 g/kg. Table 4 shows that the addition of formic acid reduced the formation of lactic and acetic acid at DM 430 g/kg, but not at DM 200 g/kg, compared with the untreated control silage. The concentration of total acids in DM 430 g/kg silages was too low to exert an inhibiting effect on listeria numbers.

Formic acid is widely used in low DM forages (<300 g/kg) which are difficult to ensile (e.g. low sugar content, high buffering capacity). Formic acid restricts the activity of most microorganisms in forage and that reduces the requirement for fermentable carbohydrates in the fermentation process. The lactic acid bacteria (LAB) on the crop are relatively tolerant to acidic conditions, but are affected too by the application of formic acid. However, the activity of LAB is not markedly reduced until the DM content exceeds approximately 300 g/kg (*Henderson & McDonald* 1976, *Weissbach et al.* 1977). Formic acid is therefore not the additive of choice for drier silages. The reason why we applied formic acid to the drier forage was to determine whether this treatment could exert an acidic chock on the listeria and cause a quick reduction in viable counts. However, the quantity of formic acid applied on the forage was probably too small for an immediate effect on listeria survival. The amount of acid applied was quite small compared with the amount of fermentation acids produced during ensiling, particularly in low DM silages (Table 4).

The application of LAB in combination with the large supply of fermentable carbohydrates in the fresh forage led to an intensive formation of lactic acid in LAB-treated silages. The high pH values and low levels of fermentation acids in untreated control silages might be explained by a lack of epiphytic (naturally occurring) LAB in the fresh forage and/or proportional high numbers of competing micro-organisms. The acidifying effect of the LAB application

Table 5. pH values, concentrations of undissociated (Und.) acids (g/kgDM) and listeria counts in contaminated silages after 30 days. MIC values for *L. monocytogenes* are given for comparison.

	Silages treated with:			MIC values (g/kgDM) after *Östling & Lindgren* (1993)	
	No additive	Formic acid	Lactic acid bacteria	Anaerobic conditions	Aerobic conditions
Low DM level (200 g/kg)					
pH	4.9	4.3	3.9	4.5 to 5.1	4.8 to 5.1
Und. lactic	5.8	20.7	61.3	0.36	1.44
Und. acetic	3.3	8.1	8.7	0.96	1.44
Und. formic	<0.1	0.7	<0.1	-	-
Sum of undissociated acids	9.1	29.5	70.1	-	-
L.monocytogenes (log cfu/g)	2.3	nd	nd	-	-
Medium DM level (430 g/kg)					
pH	5.8	5.5	4.1	4.5 to 5.1	4.8 to 5.1
Und. lactic	0.3	0.2	30.9	0.12	0.48
Und. acetic	0.3	0.3	4.1	0.32	0.48
Und. formic	0	<0.1	0	-	-
Sum of undissociated acids	0.7	0.5	35.0	-	-
L.monocytogenes (log cfu/g)	7.9	4.2	nd	-	-

[+]nd = not detected.

was evident at both DM levels (200 and 430 g/kg). The high production of lactic acid in LAB-treated silages led to a considerable pH-decrease which increased the amount of undissociated acids dramatically. These conditions reduced listeria numbers efficiently to below detection limit within a period of 30 days (Tables 4 and 5, Fig. 1).

Husu et al. (1990), who analysed 225 silage samples collected from 80 Finnish farms, detected *L. monocytogenes* in 19% of 165 silages treated with formic acid-based additives, in 44% of 25 LAB-inoculated silages and in 23% of 35 untreated silages. The authors stated that the number of LAB-inoculated silages was too small to compare additives and that LAB-inoculated silages in general were of poor quality. The average DM content of these silages was only 201 g/kg. It is common that low DM forages don't contain enough fermentable sugars

to complete the intensive lactic acid fermentation typical for wet silages. The lack in fermentable sugars can stimulate the proliferation of undesirable micro-organisms that are able to grow on other substrates. This might produce unstable silages with rising pH values that eventually support listeria growth.

Undissociated acids and MIC values

The antibacterial action of an organic acid towards a particular micro-organism is explained partly by its pH-decreasing action (acidity) and partly by the specific effect of the undissociated form of the acid (*Woolford* 1975, *Baird-Parker* 1980). Only the undissociated molecule of an organic acid can penetrate the cell membrane of micro-organisms and acidify the cell contents which leads to growth inhibition and eventually death (*Corlett & Brown* 1980). Fig. 1 demonstrates that the proportion of undissociated acid
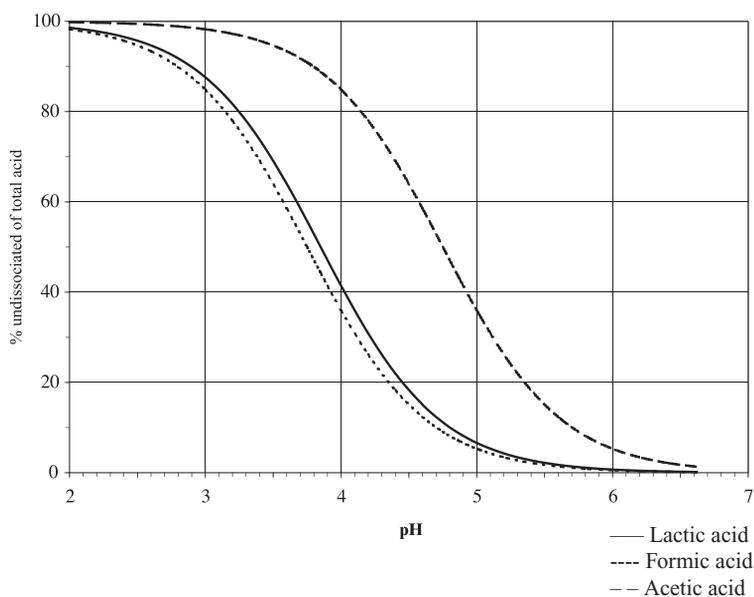
Figure 1. Proportion of undissociated acid (in % of total acid) between pH 2 and 7.

decreases rapidly as pH increases. The amount of undissociated acids in silage is therefore highly dependent on silage pH.

This study showed that very high initial numbers of *L. monocytogenes* were reduced below detection level within a month if the amount of undissociated acids was approx. 30 g/kg DM or higher (Fig. 3c). Unfortunately, no silage samples were available with concentrations of undissociated acids between 9 (listeria detected) and 30 g/kg DM (no listeria detected). If these values are compared to MIC values presented in Table 5, it appears that an acid concentration equivalent to MIC values would have had very little effect on listeria counts. *Östling & Lindgren* (1993) stated that levels of undissociated acids which frequently occur in silages with pH between 4.1 and 4.5 are about 10-100 times higher than MIC values required to eliminate *L. monocytogenes*. The reason why detri-mental micro-organisms still might be able to multiply is explained by the fact that MIC values are usually determined in liquid cultures, that is in a very homogeneous medium. Silage, especially unchopped silage, is a rather heterogeneous growth medium with respect to the distribution of moisture and fermentation acids (*Pauly* 1999). Survival of detrimental micro-organisms in silage will therefore not primarily depend on average values determined from silage samples but on the prevalence of small niches in the silage where conditions are favourable (e.g. high moisture, low acid content). For that reason MIC values determined in liquid cultures cannot indiscriminately be applied on farm silages.

Factors affecting listeria survival
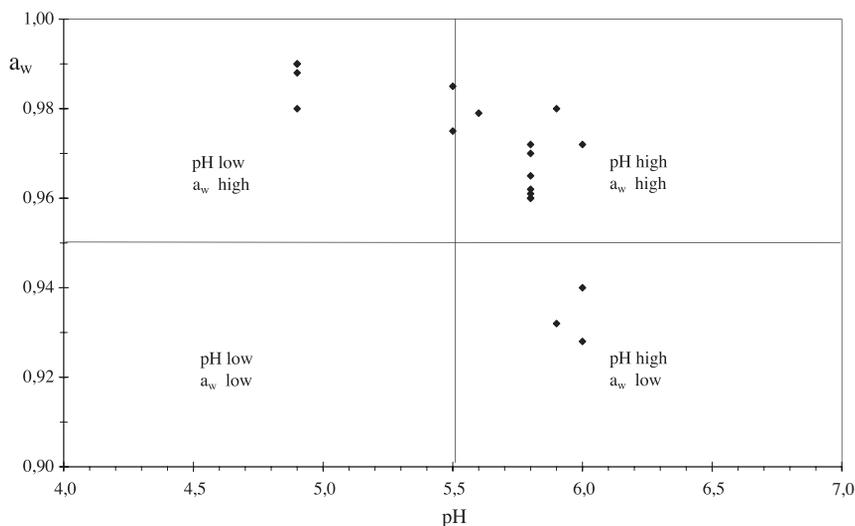For correlation and regression calculations between listeria counts and silage parameters only

Figure 2. Relation between water activity (a$_w$) and pH of silages in which *L. monocytogenes* were detected after 30 days of storage (n = 19).

data from contaminated silages after 30 days storage were used (n = 30) because no listeria were recovered from uncontaminated and 90 day-silages (except one dry silage after 90 days).

Silage pH had the strongest single influence on listeria counts (r = 0.92; Fig. 3a). According to the regression line in Figure 3a listeria counts approached zero at approx. pH 4.1. All listeria-containing silages had pH values between 4.9 and 6.0 (Fig. 2) in contrast to listeria-free silages which had pH values between 3.9 and 4.9. *Irvin* (1968) demonstrated in laboratory experiments that the growth rate of *L. monocytogenes* was static when the pH in the liquid medium was 5.5. Below this level viable counts decreased. *Gray & Killinger* (1966) and *Seelinger & Jones* (1986) stated the same pH limit. It is, however, common that results from farm-scale and laboratory experiments disagree because of the heterogeneous nature of farm silage (*Spoelstra* 1981). For example *Husu et*

al. (1990) reported that the lowest pH value at which *L. monocytogenes* was detected was pH 3.7 (N = 225 farm silages). However one can assume that the pH at the spots where listeria actually grew was much higher than 3.7. The most likely places for listeria to occur would be where air penetrates into the silage, e.g. at the surface of silages (*Fenlon* 1986a). Yeast growth is very much stimulated by the ingress of air because many yeasts are able to grow on lactic acid if oxygen is available (*Lindgren et al.* 1985). The ingress of air will therefore increase the pH in the silage and might stimulate the growth of undesirable micro-oganisms such as listeria (*Fenlon* 1986b).

Much of the variation of listeria counts in this study could be explained by lactic acid concentration (r = -0.80; Fig. 3b) and by the pooled amount of undissociated lactic, acetic and formic acid (r = -0.75; Fig. 3c). Figure 3c shows that 3 LAB-treated silages (low DM level) contained almost double as much undissociated
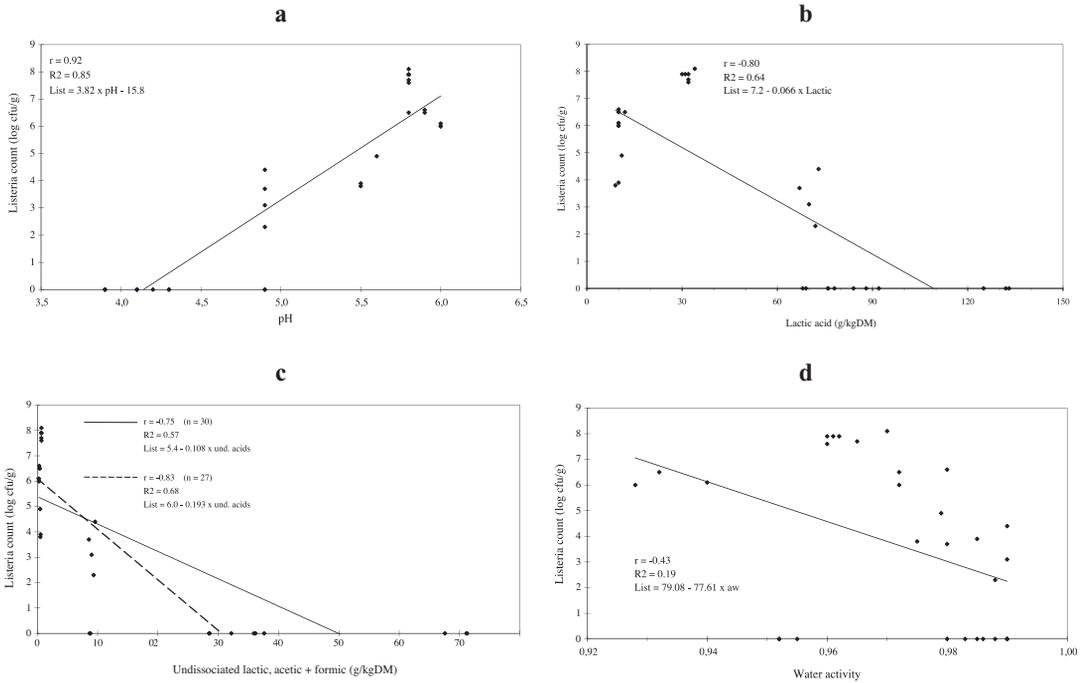
Figure 3a-3d. Correlations between counts of *L. monocytogenes* and silage constituents in contaminated silages (n = 30) after 30 days. Silage constituents: a) pH, b) lactic acid, c) pooled undissociated acids and d) water activity.

acids (68, 71 and 71 g/kgDM) than other listeria-free silages (around 35 g/kgDM). Such high concentrations of inhibiting substances do not help to explain how listeria survival is affected by these acids. When these 3 values were omitted from the data set, the correlation coefficient improved from -0.75 (n = 30) to -0.83 (n = 27). Other factors such as DM content (r = 0.67) and water activity (r = -0.43; Fig. 3d) had a much smaller influence on listeria counts. The best regression with two independent variables, pH and lactic acid, had only a slightly higher R^2_{adj} (0.88) than the regression with pH alone (0.84). R^2_{adj} measures the proportion of total variation which is explained by the regression equation. However, it should be kept in mind that samples

for listeria enumeration and water activity were taken from the upper 6 cm of the silo while the remaining contents of the silo were used for the determination of DM, pH and fermentation products (acids). Since all silos were not completely air-tight (e.g. silos with capillary tubes) and oxygen affects silage composition unfavourably, it is not unlikely that the composition of the silage had varied between the upper and lower parts of a silo. This might have blurred the results from the regression analyses. These results suggest that the concentration of lactic acid is the most important factor for the inhibition of listeria in silage since lactic acid it is generally the main fermentation product in silage. Because lactic acid is a strong acid it

controls both silage pH and the degree of dissociation of the other fermentation acids.

Fenlon (1989) investigated the effect of water activity and pH on listeria survival and showed in liquid cultures that 2 tested strains of *L. monocytogenes* were not able to grow below 0.95 a_w under aerobic conditions and not at 0.99 a_w or lower under anaerobic conditions (pH range 4.5-6.0). The growth limits for <u>aerobic</u> conditions agreed reasonably well with the results from our silos. In our mini-silos the inoculated listeria strain increased in numbers at 0.96 a_w (pH 5.80) and stayed on the same level at 0.95 a_w (pH 5.93) during the first 30 days (Table 3). Conditions in our silos were neither aerobic nor completely anaerobic because a small but unknown quantity of air could enter into our silos through the capillary tubes and to a smaller extent even through the water locks. On the other hand the conditions were not aerobic enough to support mould growth on the silage surface. A direct comparison with *Fenlon's* (1989) data is therefore difficult. In addition, *Fenlon* gives no information on the type of acidifying agent and the type of salt used to reduce water activity (according to *Shahamat et al.* (1980a) *L. monocytogenes* tolerates high concentrations of NaCl). Furthermore, it should be considered that other inhibitory factors such as temperature and nitrite concentration had been shown to affect listeria survival (*Shahamat et al.* 1980b, *George et al.* 1988). Whenever possible these growth factors should be measured simultaneously.

Conclusions

- *L. monocytogenes* was able to survive in the untreated silages (pH ≥4.9) for longer than 30 days but not longer than 90 days, except in one untreated high DM silage. An increase in storage time appears to reduce listeria counts.
- An increase in DM content up to 540 g/kg (equivalent to a water activity of 0.95) did not reduce counts of *L. monocytogenes* within the first 30 days after ensiling. On the contrary, the lower formation of acids in dry silages appeared to have a favourable effect on listeria growth.
- Both the addition of formic acid and lactic acid bacteria shortened the survival period of *L. monocytogenes* in the low DM silages (200 g/kg). In wilted silage (430 g/kg), the application of lactic acid bacteria produced a more acidic silage than the formic acid-treated silage and was therefore more efficient in eliminating *L. monocytogenes.*
- *L. monocytogenes* counts after 30 days of storage were highly correlated to silage pH (r = 0.92), lactic acid content (r = -0.80) and pooled undissociated acids (r = -0.83). Lactic acid concentration is the most important factor since it has a strong influence on both silage pH and the proportion of undissociated acids.
- The most practical way to inhibit the survival of *L. monocytogenes* in grass silage appears to be to produce intensively fermented silages and store the silage for more than 30 days. The application of an acid additive to wet silages (DM <300 g/kg) or an efficient bacterial additive to wilted crops (DM >300 g/kg) can be recommended.

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Sammanfattning

Överlevnad av Listeria monocytogenes i förtorkat och behandlat gräsensilage.

En gräsgröda som hade fälttorkats till 3 olika torrsubstanshalter (TS-halter) ympades med mellan 10^6-10^7 cfu *L. monocytogenes* per gram gräs. Den använda bakteriestammen tillhörde en fagovar som har associerats med livsmedelsburna utbrott av listeriosis. Myrsyra (3 ml/kg) eller mjölksyrabakterier ($8\cdot10^5$/g) med cellulytiska enzymer tillsattes endast till grönmassan med låg och medelhög TS-halt. Dessa partier ensilerades sedan i små laboratoriesilor (1700 ml) som förvarades vid 25 °C i 30 eller 90 dagar.

Efter 90 dagars lagring kunde inga *L. monocytogenes* påvisas med undantag för ett enda obehandlat silo med hög TS-halt ($<10^2$ cfu/g). Efter 30 dagars lagring kunde mellan 10^2 och 10^6 cfu *L. monocytogenes*/g isoleras från de obehandlade ensilagen. Den kraftiga förtorkningen av vallfodret - från ca. 200 upp till 540 gTS/kg - minskade inte listeria-antalet, vilket troligtvis berodde på att mjölksyrabildningen minskar (högre pH) när TS-halten i ensilaget stiger. I ensilagen som behandlades med ensileringsmedel var listeria-antalet alltid lägre än i de obehandlade ensilagen. I de direktskördade ensilagen (ca 200 gTS/kg) hade både myrsyran och bakteriemedlet effekt, men i det förtorkade ensilaget (ca 430 gTS/kg) reducerade endast bakteriemedlet listeria-antalet inom 30 dagar till under detektionsgränsen.

Antalet *L. monocytogenes* i ensilaget var starkt korrelerad till pH-värdet (r = 0,92), till mängden mjölksyra (r = -0,80) samt till den sammanlagda mängden odissocierade syror (r = -0,83).