Silage bacteria & toxins and ruminant health

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Introduction

If prerequisites for the desired lactic acid fermentation during silage making are not met, organisms other than lactic acid bacteria (LAB) may multiply and for shorter or longer time dominate the ensiling process. This eventually leads to the accumulation of harmful fermentation products (amines, toxins) and loss of energy as well as palatability. In addition badly-fermented or aerobically deteriorated silage may facilitate the survival or multiplication of undesired organisms that normally would be eliminated in well-fermented silage. To assess the risk if a certain undesired organism would be able to grow in silage of some kind, it is important to collect information of the environmental conditions that permit its growth. Lindgren (1991) listed minimal inhibitory concentrations (MIC) of some undissociated organic acids for some silage bacteria (Table 1). He states that the levels of undissociated organic acids in silages are more reliable parameters for microbial inhibition than the frequently used pH values. The antibacterial action of an organic acid is explained partly by its pH-decreasing action and partly by the growth-inhibiting effect of the undissociated acid on the microorganism in question (Baird-Parker 1980). The reason why Lindgren recommended to use undissociated and not total acids was because i) only the undissociated acid can pass through the cell wall, release its H-ion into the cell contents and thus reduce or inhibit growth by disturbing the micro-organism's metabolism and ii) MIC concentrations of the undissociated part of an organic acid is relatively constant within a pH range typical for

Table 1. Minimal inhibitory concentrations (MIC) of *undissociated* lactic acid (LA) against some silage associated organisms (Lindgren 1991). 10 mM lactic acid = 0.9 g/litre silage juice equivalent to 0.63 g/kg FM or 2.1 g/kg DM in a silage with 30% DM.

Organism	MIC, mM LA	Valid pH range
Enterobacteria	6-10 mM	4.2 – 5.4
E. coli	4-6 mM	4.2 – 5.4
Cl. tyrobutyricum	5-10 mM	4.8 – 5.4
Listeria monocytogenes	1-3 mM	4.5 – 5.1

silage (pH 4 – 5). The proportion of the undissociated part of an acid at a certain pH can be calculated as:

Proportion of undissociated acid (%) = $100 / (1 + 10^{pH-pKa})$

Figure 1 demonstrates that at a certain pH a much larger part of acetic or propionic acid is undissociated compared with lactic of formic acid.

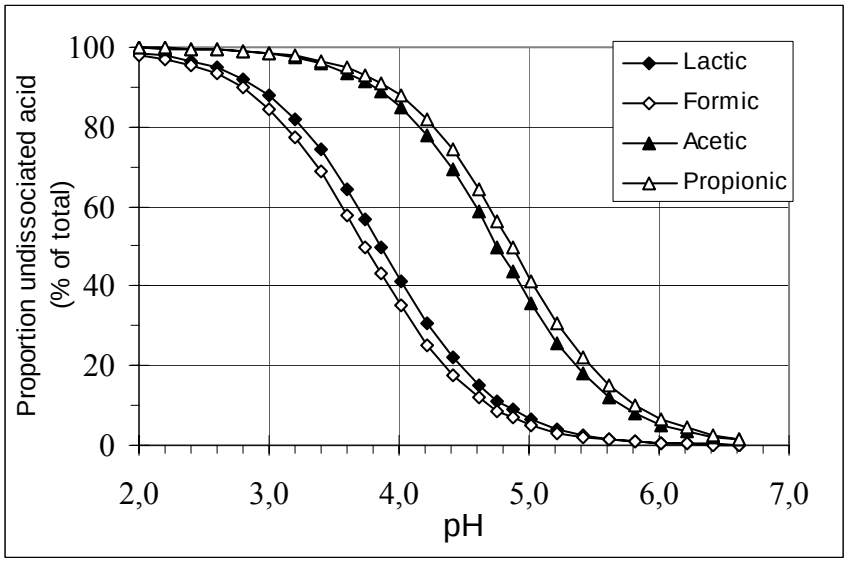

Figure 1. Proportion undissociated acid as % of total acid in the pH range between 2 and 7.

Well-fermented silages, in which fermentation is not restricted by intensive wilting (<50% DM) or addition of chemical additives, contain commonly approx. 50 -

100 mM of undissociated lactic acid. This gives the impression that we should be safe from any of the silage-associated organisms listed above. Still, we frequently find badly fermented silages, which contain concentrations of undissociated LA that are much higher than the MIC values listed in Tab.1. The reason why MIC values, elaborated in carefully controlled lab experiments, often appear to be too low for full-scale silages might be explained by the heterogeneous nature of farm-scale silages (Spoelstra 1982, Pauly 1999). Silage samples collected from a farm silo might contain excellent silage together with small chunks of badly fermented silage. The example in Table 2 demonstrates how we can get such a proliferation of clostridia (log 5.1/g) in silage that contains 18 g of undissociated lactic acid per kg, a concentration that should inhibit the growth of clostridia with large safety margins.

Table 2. Example of a silage sample split into 10 small sub-samples. The composition of the composite sample (Total) would reflect the results from the lab analyses.

											Total
Fractions of the sample (1-10):	**1**	2	3	4	5	6	7	8	9	10	**18**
Undissociated lactic acid, g/kg:	**1**	22	20	20	19	21	18	20	22	18	**18**
Clostridial spores, log cfu/g:	**6.1**	2.0	2.2	2.1	2.1	2.0	2.3	2.0	2.0	2.1	**5.1**

Sometimes differences in silage composition are indicated by a divergent colour or odour, like when air leaks into a silo. Then we can respond to that and avoid or select these zones when we collect our samples. However, if we cannot sense any difference in colour or smell, we have not a clue where in the silo these badly-fermented pockets of silage are and how large these niches might be. When inhibiting growth conditions for undesirable silage microorganisms are discussed, we should be aware that we need to add safety margins to the values gained from lab experiments. Safety margins should be chosen in relation to the expected heterogeneity in the examined silage. A heterogeneous silage like most wilted bale silages would probably require larger safety margins than shortly wilted, precision-chopped bunker silage.

As the example in Table 2 shows it is common in silages that fermentation products and bacterial counts appear to disagree with another, like when bacterial counts are high but their fermentation products low or vice versa. This is because the activity of silage bacteria changes over time while fermentation products accumulate. Prerequisites for the production of well-fermented silage are sometimes summarised with the term ´good ensiling practices'. For more detailed information on silage making see McDonald et al. (1991) and Al-Amoodi et al. (2003).

Clostridia in silage

Clostridium species

Clostridia are Gram-positive, usually motile, spore-forming bacteria, which have their natural habitat in soil and in fresh- or saltwater mud. They are even found in intestinal tracts of mammals and fish and the gills and viscera of shellfish. Most species require icroaerophilic or strictly anaerobic conditions for growth and multiplication. Due to the lack of specific enzymes found in most aerobic microorganisms (superoxide dismutase, catalase) oxygen is toxic to them. When growth conditions are unfavourable, i.e. in the presence of oxygen, clostridia transform quickly from the active viable form into the inactive spore form. Thick cell walls of spores make them very resistant to adverse environmental conditions such as disinfectants, irradiation, drying or heat (Madigan et al. 1997). As spores they can survive for decades in a kind of stand-by stage in which their metabolism is reduced to a minimum. Spores cannot multiply. Only when spores germinate and form viable cells, counts might increase and exotoxins might be released.

Toxin forming *Clostridium* species

Bergey's Manual (Cato et al., 1986) lists 84 *Clostridium* species. Several of them are known to infect farm animals and man and can cause severe damages due to their ability to produce powerful toxins. The following three clostridia species cause enteritis, paralysis or tissue necroses but are not associated with silage feeding and will therefore not be discussed further.

Cl. perfringens	enteritis, tissue necroses from puncture wounds
Cl. difficile	enteritis or enterotoxaemia
Cl. tetani	muscle paralysis and spasms from puncture wounds

Cl. botulinum is the only toxin-forming species that occasionally is connected with silage feeding, usually deteriorated or decayed silage. In many regions *Cl. botulinum* is closely associated with extensively reared cattle chewing bones of dead animals to obtain phosphorus or protein. In endemic areas, type-specific vaccines and antitoxins are used to reduce or treat cases of botulism. Seven or eight different strains are known (A to G), grouped after the type of toxin they produce (Wilkins 2007). Different animals are sensitive to different types, e.g. cattle are mainly affected by type A, B, C and D, horses mainly by type B and humans by type A, B, E and F (Iowa State Univ.: Botulism Fact Sheet). Pigs and cats are relatively resistant to botulism. Notermans et al. (1978) and Roberts (1988) said to have found evidence that *Cl. botulinum* can multiply in the gastrointestinal tract of cattle or man and may continue to produce toxin. Botulinum toxins (BT) are proteins or polypeptides and are among the most powerful neurotoxins known. They block the release of acetylcholine, which makes a muscle contract, and cause progressive paralysis of muscles. Notermans et al. (1979) estimated that 10 to 100 g of highly contaminated silage may contain sufficient BT to kill a cow. The same authors tested in total 11 strains of *Cl. botulinum* (type A, B, C and E) and found that only the proteolytic strains of type A and B were able to produce toxin with only grass as substrate. This supports the fact that an animal protein source (such as a dead animal) is not a prerequisite for BT production in silage. *Cl. botulinum* reacts more sensitive to low pH levels than the typical silage clostridia (see below) and will therefore not grow in well-fermented silage. Never the less this species may at times survive and multiply in pockets in the silage where pH and water activity are high enough to permit growth. That could be in pockets with aerobically deteriorated or soil contaminated silage or inside a small animal (intestines) that was killed during cutting. The carcass must be large enough to protect its inside from the penetrating fermentation acids. This could be the reason why *Cl. botulinum*-contaminated insects

Picture 1. Adult specimen of *Arion lusitanicus*, 7-10 cm in

Picture 2. Grass ensiled with slugs in laboratory silos.

or invertebrates in silage do not appear to pose a threat to ruminant health but larger animals like cats, rabbits or deer calves might do.

In 2007 we had problems with a massive multiplication of slugs on leys on the west coast of Sweden. The slug was a new, invasive variety (*Arion lusitanicus*) from central Europe (Picture 1) and because of its cannibalistic habits it was called 'murder slug' by media and public. During the same year six calves died in this area and dairy cows refused to eat the slug-infested silage. Soon speculations were raised on the possible involvement of *Cl. botulinum* and farmers demanded quick actions from advisers and researchers. The year after we got economic support to make a preservation experiment in laboratory silos with an untreated control (no slugs) and 3 levels of slugs (corresponding to 20, 40 and 80 slugs/m^2) mixed into a grass crop with a DM content of 33% and 53%. Quite opposite to what was expected, the results indicated an advantageous effect of the slugs on fermentation characteristics of the resulting silages. The slugs contained relatively high counts of LAB, which increased the formation of lactic acid. Counts of *Clostridium* spp. and enterobacteria were below detection level (<10^2 and <10 cfu/g FM, respectively). DNA sequences from *Cl. botulinum* type C (PCR technique) were found neither in slugs nor silages. But even if the fermentation quality was excellent, cattle might still find slug-infested silage not very palatable.

Fertilizing forage crops with poultry litter, which often contains decayed carcasses, or mixing poultry litter with forage crops to increase the crude protein content of the resulting silage is common practise in some countries (Otter et al. 2006, Sharpe et al. 2008). However, that is a very risky operation because if mixing is uneven, air is leaking into the silo or acidification in some part of the silage is insufficient, an outbreak of botulism, usually with high mortality, would be highly likely. In addition it should be considered that toxins formed in poultry carcasses before ensilage cannot be destroyed by fermentation acids in the silage.

Sometimes cattle are forced to eat decayed or soil-contaminated silage because no other forage is available to them, e.g. when low-rank cows do not dare to approach the feed source where high-rank cows linger. But that is, strictly speaking, not a silage but a management problem.

The dry matter (DM) content of the silage might play an important part too, because the pH of the silage increases with increasing DM content (Morgan et al. 1980, Pauly & Tham 2003). High DM silage might therefore be a risk factor

depending on if the water activity (a_w) is low enough to inhibit growth of *Cl. botulinum*. Notermans et al. (1979) state that *Cl. botulinum* type A and B needed for toxin production a minimum water activity (a_w) of 0.94 at pH 5.8 - 6.5 and at least 0.985 a_w at pH 5.3. In theory DM content and water activity of a silage crop should be highly correlated to each other (negatively) but according to our experience with grass-based silage crops this correlation varies too much between cuts and years to estimate a crop's water activity from its DM content. Other crops might respond more predictable and it might be easier to state a safe DM range, which does not permit *Cl. botulinum* to grow. But if we deduct from Notermans et al. (1979) data that silage with a pH below 5[1] should be safe to feed, we can conclude that that could be easily achieved if we do not wilt the forage crop above 40-45% DM, possibly add a LAB-based additive and apply what is generally called 'good ensiling practices'.

However, farm silage is often not very homogeneous and pockets with deteriorated silages might exist right next to excellent silage (Spoelstra 1990). It is common practice to discard visually deteriorated silage, but not all quality flaws in a silage are discovered and occasionally pieces of decayed silage unintentionally end up in the forage that is fed. When total mixed rations (TMR) are fed, all ingredients are thoroughly mixed and animals have no possibility to avoid spots of the silage that might contain BT. On the contrary, a chunk with BT-containing silage might be distributed in the entire TMR and might affect many cows. For the vet it might prove very difficult to find the feed (or part of feed) that caused the disease. Even when blood (serum) or contents of intestines are sampled it is frequently difficult to find any BT and verify the type of organism or toxin that caused the disease.

Common *Clostridium* species in silage

Less then 10 *Clostridium* species have been isolated from silage or cattle manure (Table 1). The 4 most common species in silage are *Cl. sporogenes, Cl. tyro-butyricum, Cl. butyricum* and *Cl. bifermentans.* All of them ferment water-soluble carbohydrates (saccharolytic activity) and some of them lactate or amino acids and peptides (proteolytic activity). None of them is known to produce any toxins. Pahlow et al. (2003) state that proteolytic clostridia, which are unable to ferment

[1] Roberts (1988) states that *Cl. botulimum* is unable to grow at a pH ≤4.6, but that pH, water activity and temperature act in combination.

carbohydrates, are *not* commonly found in silage. This is probably why *Cl. botulinum* and *Cl. perfringens* are only rarely isolated from silage.

Table 3. Number of isolates of *Clostridium* species recovered from silage or cattle manure (Ali-Yrkkö et al. 1978 and Bühler 1985; after Pahlow et al. 2003).

Species	Silage	Manure
Cl. sporogenes	13	69
Cl. tyrobutyricum	51	28
Cl. butyricum	12	8
Cl. bifermentans	14	5
Cl. acetobutyricum	9	
Cl. perfringens		1
Cl. paraputrificum	5	2

When spore-containing silages are fed, the concentration of spores increases on the way through the gastro-intestinal tract about tenfold (Stadhouders et al. 1985). This is why the spreading of manure (solid) or slurry (liquid) constitutes a potential contamination risk. Large forage plants, like corn or sorghum, which are harvested without wilting and with a high stubble height (approx. 20 cm), are usually not contaminated with soil. Temperate grasses and forage legumes, which are commonly wilted in the field and cut at a low stubble height (approx. 5-10 cm), form a much larger contamination risk. Our experiences with temperate grass crops indicated that spreading of manure (solid) had a detrimental effect on silage quality, because small pieces of manure always ended up in the harvested forage where spores in the manure germinate and multiply (Rammer 1996). Spreading of slurry (liquid) was acceptable, if it was applied on the stubble right after the cut (i.e. no soiling of plants). Slurry injection in the soil was acceptable if the soil was moist and soft, but a dry and hard soil caused splashing of slurry and contaminated the plants and eventually the silage crop (Pauly & Rodhe 2001).

The clostridial spores commonly found in silage do not pose any health risk for man or animal and they do not produce any toxins. Butyric acid and ammonia, typical products of clostridial fermentations and easily identified by their pungent smell, should not be a problem for cattle because they produce large amounts of it in their

rumen. Then why should clostridial fermentations be avoided? Many farmers have experienced reduced silage intakes and decreasing weight gains or milk yields when clostridial silages were fed (McDonald et al. 2002). The main reasons why farmers should try to avoid any clostridial fermentation in their silage are:

- Clostridial fermentations lead to the production of H_2 which means that a large part of the energy content in the fresh crop is lost (approx. 18% of gross energy according to McDonald et al. 1991, p.242).
- Clostridia are able to ferment the amino acids that arise from their own proteolytic activity and the action of plant proteases. Amines, carbon dioxide and isobutyrate are produced by decarboxylation of amino acids and ammonia and organic acids by deamination (Rooke & Hatfield 2003). The production of toxic amines[2] is believed to be one of the main causes for the low DM intakes of clostridial silages (van Os 1997, Neumark & Tadmor 1968). In addition, the detoxification of ammonia and probably even amines in the ruminant is an energy-demanding process (McDonald et al. 2002).
- Dairies, which produce certain types of hard cheese, can expect economic losses when cheeses slowly inflate during storage due to excessive gas production (CO_2, H_2) by clostridia (mainly *Cl. tyrobutyricum* and *Cl. butyricum*) (Bergère & Accolas 1985). Many European dairies motivate farmers to deliver spore-free milk by reducing the payment if spore counts in milk rise above a given threshold value (e.g. 700 spores/litre).

Hence the main objective during silage making should be to take measures that inhibit or reduce the multiplication of clostridia in silage. In well-fermented silages the spore level will, remain on the same level as in the fresh crop because if growth conditions for clostridia in silage are unfavourable (pH <4.6, water activity <0.94, nitrate >2 g/kg DM) spores are not likely to geminate. The most important measures to inhibit clostridia growth are:

- Avoid contamination of forage with soil, faeces, animals (vertebrates) or decayed silage.
- Wilting decreases water activity, which will reduce or inhibit clostridial activity. Under controlled conditions clostridia have problems to multiply above 30% DM in

[2] Amines: cadaverine, putrescine, tyramine, histamine.

grass silage (McDonald et al. 1991). Taking the heterogeneous nature of farm silage into account, a DM content of at least 40% should be a safe level for most practical situations (Jonsson et al. 1990).

- When 40% DM is not achieved, clostridia-inhibiting additives can be applied. Such additives contain nitrate or nitrite, benzoate or other clostridia-inhibiting compounds as active ingredients.
- Ingress of air (oxygen) into the silo favours yeast and mould over LAB growth and tends to increase counts of clostridial spores (Jonsson 1991). The latter appears to be a contradiction because clostridia are known to grow and multiply only in an anaerobic environment. However, yeasts and moulds can metabolize lactic acid with the help of oxygen and thus create anaerobic niches with a high pH, in which clostridia might thrive. With a short length of chop, thorough consolidation and a tight silo cover, the quantity of air leaking into the silo can be minimized. In addition, these measures will help to reduce problems with aerobic deterioration (heating) when silages are fed out.

Inoculation of forage with *Clostridium* strains

Farmers should always try to minimize the spore contamination of forage crops. However, for researchers the inoculation of the fresh crop with clostridial spores might be a useful tool to challenge the ensiling process, e.g. when examining the inhibiting effect of silage additives or when clostridial fermentations are studied. When testing silage additives, it is imperative that the untreated control is going to ferment badly otherwise the potential quality-improving effect of an additive cannot be examined. The challenge for the researcher is to choose the right level of deteriorating actions so that the controls are negatively affected without turning *all* treatments bad. In some studies clostridia-containing soil was used as a spore source, but that would not test the clostridial influence *per se* because soil contains a vast range of other microorganisms plus buffering minerals and organic compounds, which all might interfere with the ensiling process. The best choice would be to add an aqueous suspension with clostridia spores to the forage crop. The question would be which species or strains and which inoculation level should we choose? In Sweden we used during the last 20 years a pure *Cl. tyrobutyricum*-strain selected from blown hard cheese and applied it at a rate of approx. 10^3 viable spores per gram of fresh forage (Pauly et al. 2008). However, in other parts of the world other

Clostridium species and strains and other inoculation rates might be more relevant. By collecting samples from badly fermented silages or milk products and by identifying the species, the most common and competitive *Clostridium* species could be identified. To culture and store selected strains is not difficult and can be done with basic lab equipment. The spore suspensions will keep for years. It is however important to assess the viability of the spores in the suspension before the experiment is performed because viability might vary within a wide range. Viable spore counts per mL of suspension are determined by making a few tenfold serial dilutions and culturing aliquots of the dilutions on *Clostridium*-selective agar plates (e.g. reinforced clostridial agar) in anaerobic jars.

Bacillus spp. in silage

Like clostridia *Bacillus* spp. are Gram-positive, endospore-forming bacteria usually found in soil, dust, manure and bedding material of farm animals. They are distinguished from clostridia by their aerobic growth (catalase positive). Some bacilli, like *B. cereus*, are facultative and can grow in anaerobic environments too. Like clostridia, high temperatures encourage bacilli to germinate and grow (Gibson et al. 1958).

The two following *Bacillus* species are well-known, but are here only briefly mentioned because none of them is associated with silage or silage feeding. *B. anthracis* is a soil bacteria that can produce a very powerful toxin that causes anthrax in humans and animals, often mentioned when biological warfare is discussed. *B. thuringiensis* forms upon sporulation crystals of proteinaceous endotoxins, which when ingested are pathogenic to many insects. This species is used for the production of biological insecticides and insect-resistant genetically modified crops.

Table 4. *Bacillus* spp. found in deteriorating silages (after McDonald et al. 1991).

Aerobic bacilli	Facultative anaerobic bacilli
B. sphaericus	*B. cereus*
B. lentus	*B. licheniformis*
B. firmus	*B. polymyxa*

B. cereus is known to be very versatile. It can grow from 4-5° up to 50°C and has been demonstrated over a pH range of 4.9 to 9.3 (Jay et al. 2005). It can cause infections of the skin, the eye or of the intestinal tract of humans or farm animals

(U.S. Food and Drug Administration) as well as mastitis in cows (Schiefer et al. 1976). Upon intestinal infections it can produce 2 types of toxins, which cause either a diarrheal type or a vomiting (emetic) type of illness (Jay et al. 2005). In dairies this species is well-known for its ability to survive pasteurization (as spores), to attach to surfaces and to cause coagulation (clotting) of fresh milk and cream (te Giffel 1997).

Some facultative anaerobic bacilli might play a minor role in the ensiling process as they can produce lactate, acetate, butyrate, ethanol, 2,3-butanediol and glycerol (Pahlow et al. 2003). Woolford (1977) tested the activities of 3 bacilli (*B. coagulans, B. licheniformis, B. polymyxa*) in grass silage with 14% DM and 8% water-soluble carbohydrates in DM. He summarized that these bacilli were not suppressed by organic acids or a low pH and had the ability to compete with LAB. However, only *B. coagulans* was capable to produce lactic acid and contributed slightly to a reduction in silage pH, but LAB were more efficient than bacilli in reducing silage pH. The tested bacilli contributed little to the fermentation and LAB dominated the silage fermentation. Moran et al. (1993) inoculated big bale silage made from ryegrass (28% DM) with a mixture of *L. plantarum, Serratia rubidaea* (an enterobacteria) and *B. subtilis*. When bales were assessed for mould occurrence approx. 3.5 months later, mould scores were lower for bales treated with the inoculant mixture than with the *L. plantarum* inoculant or the untreated control.

Strictly aerobic bacilli can survive the anaerobic storage period as spores and might germinate when silos are opened and air penetrates into the silage. They might therefore play a role during the later stages of aerobic deterioration in silages together with facultative anaerobic bacilli. They appear not to initiate the deterioration process, this is usually done by lactic acid-assimilating yeasts (Lindgren et al. 1985, Jonsson 1989) or acetic acid bacteria (Spoelstra et al. 1988). However, they are often observed at later stages in the aerobic deterioration process when silage temperature exceeds 45°C (Lindgren 1991). In moist hay they might be involved together with actinomycetes in the initial microbial heating that later might escalate and eventually, by chemical processes, leads to the spontaneous combustion of hay (Scott & Mercer 1997).

In conclusion, *Bacillus* spp. play a minor role in silages but deserve some attention on dairy farms (udder hygiene at milking), because of their negative implications to the dairy industry. Some *Bacillus* strains might become valuable

inoculants, particularly for high-DM silages, in the future because of their ability to produce specific bacteriocins or antibiotics.

Enterobacteria in silage

Enterobacteria are rod-shaped, motile, Gram-negative and facultatively anaerobic bacteria belonging to the group of *Enterobacteriaceae*. They constitute the prominent part of the gut flora in the intestines of most warm-blooded animals and are found in manure, soil and on forage crops. Sometimes the term 'coliform bacteria' or 'coliforms' is used, but that is by definition a subgroup that ferments lactose, a trait not very useful for forage plants, which lack lactose (Pahlow et al. 2003). It is now common practice (as suggested by Seale et al. 1990) to culture enterobacteria on the less selective violet red bile *dextrose* agar (VRBD) instead of violet red bile agar (VRB) that contains only lactose as the main energy source.

Enterobacteria are generally not regarded to be pathogenic but like all other Gram-negative bacteria they contain an endotoxin (lipid A) in their outer membrane (Lindgren 1991). When enterobacteria are digested and dissolve, the endotoxin is released and absorbed through the mucous membranes of the gastrointestinal tract into the blood stream. Large quantities of endotoxin are present in the rumen. There is evidence that the endotoxin concentration increases when concentrates are added to a hay diet (Haubro Andersen 2003). Cattle should be naturally adapted to this continuous flow of endotoxin, but large variations in susceptibility to endotoxin are reported. Healthy cattle appear to have mechanisms that can deal with the endotoxin, but animals with infected organs (udder, uterus) or enteritis might be more susceptible/vulnerable. The toxicity of the endotoxin might therefore be determined primarily by the health status of the animal rather than by the properties of the endotoxin *per se* (Haubro Andersen 2003).

There is a general concern that pathogenic species or strains of enterobacteria, such as *Salmonella* spp., *Klebsiella aerogenes* or *E. coli* O157:H7, might survive the ensiling process and spread from farm animals to man or vice versa (Lindgren 1991, O'Kiely et al. 1999). The same might be relevant to antibiotic-resistant strains, but tolerance to antibiotics has no competitive advantage in silage and these bacteria would probably not persist longer in silage than other enterobacteria. However, silage is a very variable substrate with a wide range of acidity and water activity depending on forage crop, DM content and maintenance of

anaerobiosis in the silo. Single ensiling experiments can only give an indication on the survival of a particular organism under particular conditions prevailing in a particular type of silage. Lab experiments that determine an organisms tolerance to fermentation products, pH and water activity are probably more useful for assessing the chance of survival in different kinds of silages. With respect to the heterogeneous nature of most farm silages, a safety margin should be added to the limiting growth conditions, which are determined in well-controlled lab experiments.

Enterobacteria are a large and versatile group of bacteria, which can, depending on the species, ferment a wide range of carbohydrates and give rise to fermentations products such as lactate, acetate, formate, ethanol, acetoin[3] and 2,3-butanediol[4] (Rooke & Hatfield 2003). Enterobacteria are sometimes divided into butanediol-producers and mixed-acid-fermenters. The latter group produces mainly acetate, formate and ethanol from glucose. They are not known to form larger quantities of acids and their capacity to acidify silage is therefore limited. Spoelstra (1985) states that they are inhibited by a pH <4.5, but points out that their ability to reduce nitrate (NO_3) to nitrite (NO_2) and further to nitrous gases (NO, NO_2, N_2O) is important for the ensiling process because nitrite and NO and NO_2 are very effective in inhibiting the multiplication of clostridia. Nitrate and the final product of the nitrate reduction, ammonia, have no inhibiting effect on clostridia. Well-fermented silages, which are made of forage crops low in nitrate often contain small quantities of butyric acid, which is produced by clostridia in the beginning of the ensiling process before a low pH inhibits their activity. The addition of nitrate to a forage crop low in nitrate usually results in butyric acid-free silages (Weissbach et al. 1993). The addition of nitrate or nitrite is advisable if the forage is ensiled below a DM content of approx. 35% DM and if the crop really is low in nitrate, e.g. has received low dressings of nitrogen fertilizer. Commercial silage additives, which have been used in Scandinavia for decades, can add between 400 g and 900 g of $NaNO_2$ per metric tonne of fresh matter (FM). Care must be taken to avoid any nitrate overdose because cattle might respond with reduced intake, poor growth and bad fitness. If nitrite is absorbed into the bloodstream, hemoglobin is converted to methemoglobin, which looses its ability

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[3] Acetoin has a 4 C structure with a carbonyl group (=O) on the 2nd C and a hydroxyl group (-OH) on the 3rd C. It has a pleasant buttery odour and is used as flavouring agent in bakery products.

[4] 2,3-butanediol has a 4 C structure with one hydroxyl group (-OH) each on the 2nd and the 3rd C.

to transport oxygen. Negative health effects might be expected if the daily nitrate intake exceeds 10-20 g/100 kg body weight (Undersander et al.).

Apart from their ability to reduce nitrate, high numbers of enterobacteria are not desired in silage because:

a) enterobacteria compete with LAB for fermentable carbohydrates during the initial stages of the ensiling process; if the content of soluble carbohydrates is low, LAB might be inhibited by the lack of fermentable substrate;
b) enterobacteria have, like clostridia, proteolytic activity, meaning they degrade protein into amino acids and peptides (Spoelstra 1983);
c) enterobacteria degrade nitrate and deaminate amino acids, which will produce ammonia that increases silage pH (Spoelstra 1987).

The number of enterobacteria found on temperate forage crops at cutting might range between 10^3 – 10^6 cfu/g FM (Pahlow et al. 2003). During the initial phase of ensilage numbers often increase up to 10^8– 10^{10} cfu/g FM (Rammer 1996), but then drop quickly after few days below detection level (Heron et al. 1993, Pahlow et al. 2003). The enterobacteria that dominate on fresh temperate grasses were often *Erwinia herbicola* (identical to *Enterobacter agglomerans*) and *Rhanella aquatilis*. After ensiling their numbers dropped fast and *Hafnia alvei* and *E. coli* dominated for a short time before they disappeared altogether (Heron et al. 1993). The rise and fall of enterobacteria in silage is very closely connected with the development of LAB. LAB numbers in the fresh crop are on average 100 times lower than enterobacteria counts (Pahlow et al. 2003), but when there is no shortage in substrate and good ensiling practices are applied, LAB will outcompete enterobacteria within a matter of days due to their better adaptation to anoxic and acidic environments. In corn silage, which has a low buffering capacity and contains plenty of fermentable substrate, the pH drop is faster than in most grass-based silages and enterobacteria are detectable only during the first day after ensilage (Pahlow et al. 2003). It is therefore not common to find enterobacteria 2-3 months after ensiling when silos are opened for feeding. However, enterobacteria might persist on spots in the silo that are contaminated with chunks of soil or manure or where air leaks in. But such silage is usually dark and smells bad and no conscientious farm worker would feed it.

Listeria in silage

Listeria monocytogenes is the causative organism of listeriosis, a disease, which affects both a wide range of animals and man (zoonosis). Six different listeria species are known but only *L. monocytogenes* is known to cause listeriosis. Typical manifestations of listeriosis are septicaemia and/or affection of the central nervous system (meningitis), abortions and occasionally mastitis (Waak 2002). Among farm animals sheep and goats appear to be particularly susceptible to listeriosis (Low & Renton 1985, Wiedmann et al. 1994).

Human listeriosis is a relatively rare food-borne disease, approx. 4-8 cases occur each year per 1 million persons (FDA, USDA & FSIS 2001). A distinction is made between infections limited to mild, flu-like symptoms (listerial gastroenteritis) and those that are severe and life-threatening (listeriosis). Although the number of human listeriosis cases is low, the mortality might be as high as 20-40% (McLauchlin 1997). Ill persons with a compromised immune defence, pregnant women, newborns and old people are particularly susceptible. The type of food that is most closely associated with outbreaks of human listeriosis is meat spreads (pâté) and Deli meats, smoked seafood, milk products (particularly unpasteurized soft cheese) and other refrigerated ready-to-eat foods (FDA, USDA & FSIS 2001). The ability of *L. monocytogenes* to grow at low temperatures makes it particular important in relation to food poisoning (Table 5).

L. monocytogenes is a rod-shaped, Gram-positive bacterium, which is spread world-wide and can be found in low numbers (<30/g) in sewage water, soil, herbage and faeces (Fenlon 1989, Weis & Seeliger 1975). Since the ’60 listeriosis has been associated with silage-feeding, in

Table 5. Growth limits for *L. monocytogenes* according to ICMSF (1996).

Growth factors	Minimum	Optimum	Maximum
Temperature (°C)	-0.4	37	45
Water activity	0.92	0.97	-
pH	4.4	7.0	9.4

particular poor quality silage (Seeliger 1961, Gray & Killinger 1966). 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 et al. 1989). Farm silos and big bales are often not completely gas-tight. If the silage is not properly consolidated and sealed or if the plastic cover is damaged, the ingress of air will stimulate facultative anaerobic organisms like yeasts. Lactate-assimilating yeasts are able to metabolize lactic acid in the presence of oxygen. That consumes the oxygen, increases silage pH and eventually facilitates the growth of undesired organisms like listeria or clostridia (Lindgren et al. 1985). Listeria counts were noticed to be particularly high in the aerobically deteriorated parts of the silage and when the deteriorated parts were removed, the dose of *L. monocytogenes* in silage could be significantly reduced (Fenlon et al. 1989). Infection of animals or man is dependant on the number of ingested *L. monocytogenes* and on the immunological status of the individual (Gray & Killinger 1966). Because listeria counts in the fresh herbage are usually too low to cause infections in healthy animals, the most important task at silage making would be to avoid the multiplication of listeria in silage. Husu et al. (1990) examined the occurrence of *Listeria* species in 68 grass and 225 silage samples (average DM 21%) collected from 80 Finish dairy farms. *L. monocytogenes* was found in 38% of the grass samples, 16% of the silage samples and at least at one occasion on 34% of all farms. No signs of clinical listeriosis were detected in any of the herds probably because listeria counts were low (counts not determined) and animals in good health. Ensiling experiments with *L.* monocytogenes-inoculated forage (10^6-10^7 cfu/g FM) at 21%, 43% and 54% DM showed that the most important environmental factors for the fast elimination of listeria was a) a pH below approx. 4.5 and b) a storage time longer than 30 days (Pauly & Tham 2003). Decreasing water activity reduces the ability of listeria to grow at low pH (Fenlon 1989). The reduction of water activity by wilting from 0.99 (21% DM) to 0.95 (54% DM), which cohered with a pH increase from 4.9 to 5.9, could not reliably eliminate all listeria within a storage period of 90 days. However, the application of an efficient LAB inoculant increased the content of lactic acid and eliminated all listeria within 30 days (Pauly & Tham 2003). To get a reliable pH reduction from a LAB inoculant, the fresh forage must contain at least 15-25 g water soluble carbohydrates per kg FM (Pettersson 1988, Pahlow 1990). At DM contents above approx. 45% only osmotolerant LAB should come into use and few if any inoculants are efficient at DM contents above 55%.

The most important measures to avoid multiplication or survival of listeria in silage are:

a) prevent ingress of air into the silo, which implies that the silage should be well consolidated and the integrity of the silo cover must be maintained;
b) if there is a risk for listeria contamination, an intensive lactic acid fermentation should be stimulated, e.g. by a short wilt up to approx. 30-40% DM and the application of an effective LAB inoculant.

In conclusion, when good ensiling practices are applied and the silage is properly sealed and stored for at least 2-3 months, the risk that some listeria might survive the ensiling process should be very scarce. To eliminate all listeria during the storage period in the silo is important because surviving listeria might be able to multiply when silos are opened for feeding and air can penetrate the silage.

Conclusions

Many of the pathogenic bacteria mentioned above can be restricted or inhibited in silage by what is generally known as ‘good ensiling practices’. The most important measures are:

- Contamination with soil, manure, animals (vertebrates) or decayed forage is reduced to a minimum,
- the forage is wilted to a level that guarantees sufficient supply of water-soluble carbohydrates for an unrestricted lactic acid fermentation (Weissbach 1996),
- the forage is chopped, consolidated and sealed in such a way that ingress of air (oxygen) during storage is reduced to a minimum,
- the silo is not opened for at least 2 months after ensilage to outcompete or reduce numbers of undesired bacteria,
- the removal rate from the silo is at least 2.0 m per week to reduce the risk of aerobic deterioration during feed-out.

The problem with silage making is often that technical aspects and logistics receive a lot of attention while microbiological aspects appear to be invisible because they are much more difficult to grasp, e.g. the assessment of contamination risk or how well the silo is consolidated and sealed. It is human that we tend to repeat mistakes from previous years when all our attention is focused on to fill the silo at the shortest time possible. With respect to the consequences of what a silo filled with badly-fermented silage means for the coming feeding period, more farmers should consider to engage a silage specialist, who helps to plan and perform the ensiling work at site (i.e. on farm). Small changes in the planning can many times work

miracles, e.g. to ensure that there are enough people left (often late at night) when the silo has to be sealed. Three aspects are particularly important when it comes to question on how the survival and multiplication of undesired silage bacteria can be avoided:

a) The maintenance of anaerobiosis in the silo, because ingress of oxygen eventually creates niches or zones of aerobically degraded silage that facilitate the survival of many undesired bacteria.
b) Efforts should be made to increase the level of homogeneity in the silage during silo filling, e.g. by trying to reduce or level out DM variations after wilting or by avoiding contamination with soil or manure particles.
c) If the undesired bacteria cannot be eliminated reliably with good ensiling practises, the application of an effective additive should be considered. It is only meaningful to apply an additive if the selected additive has a documented activity against the organism in question and if the additive can develop that activity at the targeted DM content.

With these suggestions in mind the risk for the development of deleterious bacteria in silage should be very slim.

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