A LITTLE FRESH AIR: FUNGAL TOXINS AND SILAGE

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ABSTRACT

Silage, whether cereal based or formed from other feedstuffs, is a nutrient dense, high moisture commodity that provides an excellent substrate for the growth of molds and subsequent synthesis of mycotoxins. The primary control for the latter is the low availability of oxygen for these obligate aerobes in a milieu that is thought of as being anaerobic. The problem for commercial dairies is that silage is not totally anaerobic; some infiltration of air creates micro-environments with adequate oxygen to support growth of common molds. Ultimately, mycotoxin risk in silage is just a matter of a little fresh air! The task of the farmer is to strive for best initial quality in silage preparation and then use best management of the commodity and its storage facility to reduce the risks of de novo mycotoxin formation. However, mycotoxin contamination of silage is the rule, not the exception, and producers need to understand that those risks exist and take actions appropriate to control or minimize damage in their herds.

INTRODUCTION

From a California dairy comes this test result in late summer, 2010: in TMR, T-2 toxin – 50 ppb, zearalenone – 50 ppb, aflatoxin – 23 ppb (All West Select Sires, in-house report). From the Midwest, 2010 corn crop [tests from October 13 - 30] results with deoxynivalenol testing at 2.7 ppm plus T-2 toxin and zearalenone, and early data from Canada indicate they are experiencing in corn this crop year what the U.S. faced in its 2009 crop (Agrarian Marketing, in-house report). What might this have to do with silage in California? Fungal toxins are present in cereal grain and they are present prior to harvest. They tend to occur in a commodity in mixtures, not as single toxin contaminations. The amounts can be highly variable. They are all associated with problems in domestic livestock, and, in particular, dairy cattle.

Mycotoxigenic Molds

To understand the nature of the problem, one must know a little of the fundamental biology of the organisms that produce these toxins. Mycotoxins are secondary metabolites produced by molds. The molds we watch are part of a group of filamentous fungi in the class Deuteromycetes or "Imperfect Fungi" (lacking an identified sexual reproductive stage). While their modes of reproduction are numerous, most typically they propagate by the production of spores (conidia). From the germination of a single spore arises an organism comprised of long, colorless threads (mycelium) which constitutes the 'body' of the mold. In the environment, when something essential for growth becomes limited, typically a reduction in available nitrogen or carbon, the biochemistry of the mold switches from primary metabolism that is concerned with the

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production of cell mass (vegetative growth phase) to secondary metabolism which is associated with the production of spores for continuance of the species (sporulation). Some portions of the secondary metabolic pathways yield products for which no specific function can be attributed, although evidence exists for many possibilities including competitive advantage, precursor pools for spore development, waste products, etc. Amongst these secondary metabolites are some which prove to be harmful to one or more other species. These we call mycotoxins.

The molds synthesizing such toxins are relatively simple organisms with simple needs. There must be a source of carbon and nitrogen, available water with required amounts varying by mold species, and oxygen. They are obligate aerobes. These molds infect plants in various ways such as being vectored in via insects, borne on the wind and entering through plant pollination structures (e.g. the silk in corn), or, for some, becoming directly associated with plant tissues as the plant emerges as a seedling. The rate of growth of molds in pre-harvest plants tends to be relatively slow unless the plant is under some form of stress; then, the infection process and mold level become very high very quickly. In turn, consumption of those nutritive elements of the plant supplying the needed carbon and nitrogen leads to imbalances which stimulate sporulation and secondary metabolism. Thus, mycotoxins can and do form in growing crops. Throughout the agricultural world, there is virtually no plant that is not susceptible to some toxigenic species of mold. Mycotoxins are found in cereals, grasses, oilseeds, fruits, and nuts, to name those commodities most often associated with the feeding of cattle.

In animal feeding situations, the mycotoxin picture becomes more complex because most mycotoxins also form readily in post-harvest plant materials. Shell corn, soy meal, orchard residues, etc. are suitable substrates for further attack, typically by a different array of molds than the isolate(s) originally contaminating the growing feedstuffs, and the dynamics of toxin synthesis may also be altered. Ultimately, the TMR placed before the cow is a mixture of several ration ingredients, each with its own current (and past) microfloral profile, and each with some array of mycotoxins. For the cow it is a matter of how much of which combinations of toxins are present that determines the extent of issues that arise from consumption of the feed.

Table 1   Summary: Growth/Sporulation Requirements of Molds

Growth	$	Carbon
	$	Nitrogen
	$	Water (available)
Spores	$	C:N Change (or other limiting factor)

SILAGE

General

Molds are obligate aerobes. That means that they cannot grow without oxygen present and

generally at concentrations not very much lower than generally found in air. Silage is supposed to be a feed preservation technique dependent upon anaerobic fermentation. When materials are first ensiled, the compaction of the material, whether by its own weight in a vertical silo or by movement of heavy equipment in a bunk silo, moves most of the air (oxygen) out of the system. As microbial action gets underway, the remaining oxygen is quickly exhausted and fully anaerobic conditions are in force, or so we'd like to believe. In a lab or pilot scale silo, it is possible, even probable, to achieve this result. Under such conditions, mold growth will rapidly cease and the possible amount of de novo mycotoxin synthesis will be slight. Therefore, we might expect that the virtual sole source of mycotoxin contamination of silage arises from whatever amounts were present in the crops at harvest prior to ensiling.

The reality, however, is that on a commercial dairy scale and even with best ensiling practices in force, not all air is excluded. Also, silos and their contents are not impervious to infiltration of air, even when they are in the very best physical condition. That is not to say that silage is really aerobic, just that some seepage does occur. Take for example the physical motions which occur when a heat occurs. Small micro-environments expand and on contraction, particularly if in proximity to a prior air pocket or silo boundary, a small amount of oxygen returns. And, when considering molds and their toxins in silage, one has to think in terms of such small micro-environments. An area surrounding a few kernels of corn in silage can support sufficient mold activity to produce amounts of toxin deleterious to cows. Many years ago, as the first US research on aflatoxin was just getting underway, some investigators determined empirically that a single kernel of corn could harbor as much as 400,000 ppb of aflatoxin B1. Think in multiples of kernels and broken kernels within a bunk silo and consider that similar toxin synthesis can be occurring there. The extent of potential problems can then be easily understood. Visit any dairy with bunk silos and walk the face of the bunk. Scrape a bit away and small pockets of white mold mycelium can be found. Molds are occurring in those small pockets where adequate oxygen exists for their growth to continue.

Molds rarely occur as single cultures. Just as the profile of bacteria and yeast in silage is large at any given moment, so, too, is the population of molds, both those actively metabolizing and those existing only as viable spores awaiting appropriate growth conditions. And as conditions within silage change from temperature, other microbial activity, alterations in the macromolecules of nutrients, etc., the profile changes. Silage supporting active *Fusarium* growth at one time, may be found to be totally absent of that genus at another, yet *Fusarium* toxins are present. Table 2 provides a real-life example of this situation.

Table 2	Analysis of Ensiled Material for Dairy[1]	
Measured Variable	Result	Units
Yeasts (misc.)	120,000,000	cfu/g
Mucor racemosus	5,000	cfu/g
Absidia corymbifera	1,700,000	cfu/g
Penicillium roquefortii	700,000	cfu/g
Fusarium spp.	0	cfu/g
deoxynivalenol (DON)	2.5 ppm	µg/g
zearalenone	500 ppb	ng/g

[1] May, 1995 southern MI dairy. Agrarian Marketing Corp. Internal Test Report

The farm was reporting reduced milk production, weak/failed estrus, and morbidity in the herd. Neither veterinary nor nutritional issues were found. TMR and material from silos was sent for both biological and chemical testing. The population of yeasts found is not atypical and may reflect both a high level of benign yeasts and possibly some deleterious species. Recovered molds were speciated. *Mucor racemosus* is associated with corn, grows under moderate temperature conditions and has no identified toxin(s) although filtrates of some isolates have proved toxic in lab tests. *A. corymbifera* is similarly found in corn (and silage), also grows over a wide range of conditions, and also lacks any identified toxin(s). But both abortion and digestive disruptions are common in cattle when this mold is present. *Penicillium roquefortii* is quite different. While tolerant to widely variable growth conditions, it is a mold commonly isolated from silage, it is micro-aerophilic, which simply means it requires much less oxygen than other molds, and it is known to produce a large array of highly toxic materials. Amongst the characterized toxins for this mold are PR toxin, roquefortine C, mycophenolic acid, penicillinic acid, and patulin, plus two highly toxic alkaloids, agroclavine and festuclavine. Roquefortine C and mycophenolic acid are now thought to have very significant ramifications for dairy operations. Note, however, that no *Fusarium* was isolated from this farm. Yet, the toxin analysis showed 2,500 ppb of DON and 500 ppb of zearalenone, the two *Fusarium* mycotoxins most often isolated from dairies, and the ones most frequently correlated with poor performance and reproduction.

As a substrate for mold growth, silage is an ideal commodity since it is nutrient packed (exceptional source of carbon and nitrogen) and has plenty of water. Further, contrary to some popular opinion, molds prefer acid environments; thus, silage provides a great growth medium except to the extent that oxygen is limited.

SILAGE IN CALIFORNIA

While all the foregoing apply, some conditions are prevalent in California which are not

encountered elsewhere in the US. In the primary dairy regions, relative humidity is almost always very low. Midwest farmers may expect 40-60% RH on average, but California will more likely be in the 10-25% range most of the time. While the silage itself has high water activity, its surfaces are likely drier than would be expected elsewhere. Most likely this helps to limit the degree of externally driven infection (e.g., wind-borne spores). Also, the average temperatures are higher in this region. This does not stop or severely limit mold growth or toxin synthesis but it does have some effects. For example, the Michigan farm highlighted in Table 2 would be expected to sport a veritable rainbow of visible mold, mainly *Fusarium*, during cold winter months, on the cut face of the silage. Fusarium is considered a cold climate mold (for the record, species of this mold are regularly demonstrated to be actively growing and producing toxins in the southeast US, Asia, etc. in the midst of summer heat), since it often has a competitive advantage over some other prominent genera when temperatures are below 10C. Silage in California will, therefore, likely see somewhat different mold/mycotoxin profiles on a regular basis than might be considered standard throughout the rest of the US. For quite a while, test results on silage have shown relatively little DON and zearalenone in CA when compared to almost any other state or region.

A second factor in California is the nature of the surrounding agriculture, specifically fruit and nut orchards. Most fungal infections originate from the native mold flora existing in soil and on growing or decaying plants. The molds will tend to be somewhat 'specific' to the kind of environment; therefore, where most agriculture is concentrated in cereals, beans, etc. the mold profile will reflect that environment. Here, the kinds of molds, whether at the genus or species level, will have some differences because of the extensive orchard presence.

Ultimately what is most important to remember is that evaluation of silage for either molds or toxins may need to be undertaken in California with a different perspective than is considered standard for the rest of the US. Most locales focus on *Fusarium* and DON and/or zearalenone. Decisions about silage, and feed, quality and measures to be taken to overcome problems are driven by those analyses. Lowered frequency of *Fusarium* molds, or negative tests for DON and/or zearalenone in CA do not mean that molds and toxins are not just as prevalent. Your dairy colleagues who face the challenges of high somatic cell counts, poor production, feed wastage, sorting, irregular estrous, weak estrus, poor conception rates, abortions, etc. can attest to the fact that among the significant problems in CA dairy operations are molds and mycotoxins.

EVALUATING SILAGE FOR MYCOTOXINS AND MOLDS

Briefly, the testing of silage (and TMR, for that matter) for both molds and mycotoxins is problematic. In that early work on aflatoxin in corn in the mid-1960's, it was also determined that the analysis of the toxin in shell corn had strict requirements to reduce test error. As error increases in an analysis, the spread of values obtained on repeated analyses also spreads. What should be a sharp-peaked bell curve with a tight cluster around the mean becomes spread out. Then any single test can be off from the true value by a very great amount. It was determined that to achieve best test results with corn, about 5 kg of corn was needed in the original, composite sample. In its entirety it was coarsely ground and a 1 kg sub-sample taken that was, in turn, re-ground to pass a 20 mesh sieve. From that material, 50 gm of analytical sample would be extracted for the analysis. As sample size was reduced, either from the initial 5 kg or sub-

sample 1kg, error increased exponentially. Consider that shell corn would have a typical moisture around 12%. What, then, should be the sample size of silage? First, the material is not very uniform nor consistent in composition. Second, silage moisture is on the order of 65-70%. So, instead of 5 kg, around 25 kg or more would be needed to obtain the same dry matter. Dairymen, and others taking samples on a dairy, will typically walk the lane of a feeding barn grabbing a handful here and there to make up about 0.5 to 1 kg total. Already there is the introduction of a multi-fold error in what the test will reveal. Certainly it is well beyond practicality to collect 25 kg, and even if one were willing to do so and pay the freight to send it to a lab, no commercial test lab would accept such a sample. For almost all, drying capacity is very limited. In fact a common practice in labs is to take the 1 kg silage sample and split that further to minimize dryer space. Is all lost?

Table 3		Summary: Silage
Conditions	$	Not completely anaerobic
	$	Micro-environments with some oxygen permit mold growth
	$	Water (available)!
Mold	$	Many different species can exist in silage
	$	Range of potential toxins affecting dairy is large
CA	$	Specific molds and mycotoxins may be different in CA than in other states
	$	Climate and allied agricultural commodities likely drive differences in fungal flora
	$	Evaluation of silage may require a different approach/perspective in CA

Despite the major error associated with sampling, silage can be assessed as long as one is willing to accept the premise that results will not be absolutely accurate. We routinely advise dairymen to think not about the exact test number, but to place that result within a simple, broad-based scheme of low, moderate, or high test. For example, since we follow DON so closely in most places, 600 ppb or less may be considered a low test; 601 - 1,500 ppb, moderate; and over 1,500 ppb, a high test. Also, if a problem is present, submitting more than one sample will help reduce the uncertainty.

Finally, it is well established in analytical chemistry that as the test error increases, the analytical results will become more skewed. This is because the test cannot read below "0", or in the case of most mycotoxin tests, below the minimum detection limit (e.g., the amount shown in a result saying < 50 ppb toxin X). That means that as sample size is reduced the number of false negatives will increase. In short a 'negative' test report cannot really be taken as being negative!

PREVENTION OR CONTROL?

Unfortunately, there really is no cure for the mold/mycotoxin situation in silages. On the front end, one can take some pains to test incoming materials to minimize the amount of pre-harvest mycotoxin that gets into the silo (mycotoxins are notoriously stable; ensiling conditions will generally have no adverse effect on these chemicals). Control of the chop size and assuring maximum compaction address the issue of initial oxygen expulsion. Errors here lead to extended time for mold to grow throughout. Silage inoculants offer no real advantage for mold/mycotoxin problems. They should be used for overall speed/quality of the ensiling process but not as an attempt to control molds. The short time under most conditions for residual oxygen to be consumed as fermentation gets underway will not be affected to any great extent. Of course maintenance of the silage and its container is important. Bag-type silage enclosures must be protected against puncture; vertical silos must be kept in good condition; bunks must be properly filled, covered, etc. One of the greatest sources of mold introduction, spread, and mycotoxin formation is use of unclean or inappropriate equipment to cut silage from the bunk. The cleaner the face cut and the lower the amount of vibration behind the cut, the less mold will be distributed and air infiltrate the face. Keep in mind that once oxygen returns, molds can grow and produce concentrations of toxins sufficient to injure dairy cows within 24-48 hours. Finally, for those times when rain is present, inadequate cover on bunks leads to water flow through the silage. This may have ramifications for the concentration of certain toxins within the silage. For example, fumonisin, a *Fusarium* mycotoxin, is water soluble. Translocation from top to lower levels of the silage can occur.

Feed Preservatives

It is tempting to consider the use of the fairly inexpensive feed preservatives (mold inhibitors) used in other segments of the animal feed industry. Most are based on a blend of low molecular weight volatile acids, such as propionic and benzoic acids. Formulators of complete feeds for mono-gastric species often include these to lengthen the time taken for post-pelleting growth of mold and formation of mycotoxins. Unfortunately, the mechanism of action of these acids has little to do with the overall pH, but rather with specific ionic forms of the acids interacting with the cell membrane of the fungus. In silages, fermentation has already produced acidic conditions and some of these acids are present in amounts greater than those added to non-ruminants' rations. Even with feeds so treated, a rise in moisture will quickly begin to negate the acid effects. Thus, in a high moisture feedstuffs, such as silage or high moisture corn, the transient effects of such preservatives are already overcome. It is true, there have been times when large amounts of acid, again such as propionic, were added during the ensiling process to retard mold until the oxygen was depleted and anaerobic fermentation full underway. Generally, however, this just adds cost without producing a very positive result in terms of reducing or eliminating mycotoxin contamination. Well prepared and well managed silage has the best chance of being

low in total toxins. From there it falls to the dairyman to use the available programs for intervention and preventing the mycotoxins in silage from harming the herd.

Table 4   Summary: Silage Assessment and Mold/Toxin Control

Tests	$	Small sample size yields high errors
	$	Absolute values of test results cannot be accepted
	$	Tests should be taken as low, moderate, or high
Control	$	Quality of initial ensiling can help
	$	Best management and physical condition of storage can also help
	$	Accepting the reality and providing cows with protective programs is the primary strategy; attempting to keep silage free of mycotoxins is generally unproductive

CONCLUSIONS

Molds and the toxins they produce are ubiquitous. Since the mold is a simple organism with simple needs, trying to exclude it from agricultural feedstuffs is a wasted effort. Silage provides a nutritional base for the mold just as it does for the cow. The post-ensiling contamination of this material with fungal toxins is just a matter of a little additional 'fresh air'. Understanding that the mold will grow given its basic requirements, our attention should be focused on producing the best quality silage initially, managing it with best practices, and keeping silage storage and distribution equipment as clean and physically well maintained as possible. From there it falls to the herdsman, dairy nutritionist, etc. to include appropriate control mechanism to offset injury caused by the toxins that can and will be present.