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Potential factors that may limit the effectiveness of silage additives

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Introduction Various microorganisms, enzymes, nutritive and chemical compounds have been added to forages to maintain or improve the nutritive value of a crop as silage (Kung et al., 2003). As is the nature of most biological systems, there is considerable variation in the outcome of using these additives. This paper will briefly discuss some universal and specific challenges that these additives face in order for them to be effective.

Challenges Specific to Microbial Inoculants The viability of microbial inoculants at the time of use has been the topic of many past discussions and could be a reason why inoculants are not always effective in silages. Exposure to excessive heat and or moisture and general stability in their containers prior to mixing can be a concern if products are mishandled at various steps between manufacturing and use. For example, it is not uncommon to find half open bottles or bags of inoculants sitting in barns on farms. Care should be taken on farm to ensure that microbial inoculants are stored in cool and dry environments to protect their viability. Moisture and oxygen scavengers are used by some, but not all, manufacturers. Even inoculants that are stored frozen may be compromised if they undergo repeated freeze-thaw cycles.

Added microbes must be able to compete effectively against the epiphytic flora in forages. As an illustration of this concept, Contreras-Govea et al. (2008) concluded that *Streptococcus bovis* strain HC5 was not as effective as *S. bovis* JB1 as a potential silage inoculant probably because its growth rate was 10% slower than the latter. Environmental conditions may increase the numbers and/or change the distribution of epiphytic microbes thus affecting the fermentation process. For example, Kim and Adesogan (2006) showed that the fermentation of maize silage was adversely affected by wet conditions at harvest. Mills and Kung (2002) reported marked increases in the number of yeasts and molds in chopped barley forage that had been exposed to air in a forage wagon for 24 h prior to silo filling. Such conditions might increase the challenge faced by any added microbe. *Propionibacteria* have the capacity to convert lactic acid and

glucose to acetic and propionic acids which both have good antifungal characteristics. In theory, adding these microbes to silage should result in an improvement in aerobic stability. However, under most practical conditions, added *Propionibacteria* cannot compete in the hostile environment of a silo because they are strict anaerobes and are relatively intolerant of low pHs (Filya et al., 2006, Weinberg et al.,1995).

The temperature in the forage mass during ensiling may also affect the resulting silage fermentation and the efficacy of a silage inoculant. It is well known that temperatures above the growth optimum for bacteria have detrimental effects on their metabolism and affect their viability (Moats, 1971). At temperatures slightly above that required for optimal growth, bacteria respond to thermal stress by a rapid induction of heat shock proteins meant to help with adaptation (Gould, 1989). Kim and Adesogan (2006) reported that corn silage stored at 40°C underwent a restricted fermentation with more proteolysis and lower lactic:acetic acid ratio than silage stored at 20°C. Whereas homofermentative *Lactobacilli* grow optimally at 30 to 35°C, *Pediococcus* and some heterofermentative bacteria tend to be more thermotolerant and prefer temperatures of 40 to 45°C (Woolford, 1984). Ohmomo et al. (1996) suggested that poor silage quality and the inability of commercial inoculants of lactic acid bacteria (LAB) to be effective may be due to high temperatures (45°C or above) attained during the early stages of ensiling. Anecdotal reports from some areas in the US (e.g., Texas, California and Florida), suggest that it is difficult to make good silage under extremely hot and humid conditions. In contrast, reports of forages harvested under very cool ambient temperatures and apparently not fermenting are common from the Upper Midwestern section of the US (personal communications). Thus, cold and heat tolerance for strains of LAB used as inoculants may be important under conditions of extreme temperatures.

Numbers of LAB on forages vary considerably, but in general they number around 10^5 colony forming units (cfu) g^{-1} of wet forage (Torriani et al., 1992; Cai et al., 1999). Based on these numbers, bacterial inoculants should supply at least 100,000 (1×10^5) cfu of homolactic acid bacteria g^{-1} of wet forage in order to dominate the fermentation (Pitt, 1990). Pahlow (1991) suggested that a two-fold increase in numbers of homolactic acid bacteria was required to consistently observe a positive response to inoculation. However, using modeling techniques, Pitt and Leibensperger (1987) suggested that a 10-fold increase over existing LAB populations was required in order for an added strain of homolactic acid bacteria to be competitive in the ensiling process. In North America and Europe, most traditional silage inoculants are added to achieve a final count of 1×10^5 cfu of homolactic acid bacteria g^{-1} of forage although there are some products sold with higher counts. Products with higher counts may actually be useful because they can improve the chances that an adequate number of organisms are delivered to the crop especially in cases where accurate application rates are an issue (see later discussion on application and distribution).

Once mixed in water, silage inoculants must remain viable for the intended period of use. Research conducted by Ecosyl Ltd (personal communication) showed their strain of *L. plantarum* (MTD/1) was affected by very high levels (worse case scenarios) of chlorine (250 ppm) and hydrogen peroxide (50-100 ppm) when mixed in water for an application rate of about 2 liters/tonne. However, viability was unaffected by those levels when the organism was mixed into water to be used at a low volume rate of about 0.045 liters/tonne suggesting that there was a

protective effect when the concentration of the organism was high. Muck (1992) conducted a study on the effect of chlorinated water on the viability of several microbial inoculants. Although inoculants responded differently depending on the organism tested, he concluded that in the field, the viability of most inoculants would be fine if the chlorine level in the water used was below 0.5 ppm. Thus, water that has been treated with hydrogen peroxide or chlorinated should be monitored to ensure that toxic levels are not present when used. Recently, we highlighted the fact that the viability of many inoculants can be affected by elevated temperatures in the inoculant water tank. Although the temperatures tested in that study were not extremely high (30 to 45°C) some bacteria are more susceptible to heat stress when they are starved of nutrients (Spinks et al., 2006) as would be the case in most tanks of water. Our data suggests that water in inoculant tanks should be kept below 30 to 35°C to prevent cell death (Mulrooney and Kung, 2008). In the field, temperatures in inoculant tanks have been reported in excess of 45°C (personal communication). Placement of tanks near motors or exhausts and intense solar radiation in areas where ambient temperatures are in excess of 39-42°C may be the causes of this finding. In these situations, placement of tanks to minimize heat from machinery, use of icepacks, and insulating tanks may be warranted. Most manufacturers recommend that liquid-based inoculants be used within a 48 hr period. Although some have questioned the viability of added LAB when using pressure applicators, the pressure in used in applicator systems is probably too low to affect them.

Although “grow up” or “overnight” cultures of silage inoculants are not as popular as products that can be used on demand, proper growing conditions (for example, substrate, time and temperature) are crucial for their populations to reach a critical mass prior to use. In our limited experience, we have observed that incubation at the recommended temperature for longer than recommended times has actually reduced the number of organisms in the grow up tank. Prolonged storage of the grown up culture past recommended times could also compromise their effectiveness. Methods should be developed such that end users can monitor the numbers of organisms in the tank in a quick manner.

Because the majority of silage inoculants are LAB, these organisms are dependent on adequate amounts of fermentable water soluble carbohydrates for growth. Low concentrations of fermentable sugars can be a problem in forage crops that have under gone excessive respiration because of long wilting times because of cloudy weather and/or because they were rained on. Thus, even high levels of inoculation may be futile if there is a lack of substrate. Schmidt et al. (2005) showed that the addition of microbial inoculants added to annual ryegrass could not prevent a clostridial fermentation in forage that had been chopped but not filled until 10 hours later because there was a 54% decrease in water soluble carbohydrates during the delayed filling. This was true even when the inoculants were added to the forage at the time of chopping and before the delayed fill period.

Phages are viruses that can infect bacteria and represent one of the main causes of fermentation failure in the dairy foods industry (Mäyrä-MäKinen and Bigret, 1993). The possibility that epiphytic phages occurs in silages has been documented. Tanaka et al. (1995) reported that 25% of 77 samples of silages studied contained phages and many of them were infectious to *L. plantarum*. Kaneshige et al. (1994) showed that phage activity was responsible for failed fermentations in Italian ryegrass. Addition of *L. buchneri* did not improve aerobic stability in 2

of 5 locations in a study by Schmidt et al. (2007). Phage activity against *L. buchneri* was not detected in that study suggesting that some other mechanism was responsible for this finding. Selection of *L. plantarum* resistant to phage infection (Eguchi et al., 2000) or identifying methods to reduce phage infection of lactobacilli maybe useful in the future.

Quorum sensing is the regulation of gene expression in response to changes in the density of cell populations (Miller and Bassler, 2001). Interactions between microbes in silages via quorum sensing mechanisms has not been studied to my knowledge and there is obviously potential for quorum sensing to control some aspects of silage fermentation especially because there are major changes in populations of organisms during the fermentation process. Related to this is the fact that production of bacteriocins is controlled by quorum sensing. Bacteriocins, proteinacous toxins produced by bacteria that affect other bacteria, from LAB have been studied in hopes of controlling undesirable organisms such as clostridia and listeria (De Vuyst and Leroy, 2007). Specifically for silages, Flythe and Russell (2004) showed that a bacteriocin (bovicin HC5) from *S.bovis* HC5 could inhibit the activity of *Clostridium sporogenes* MD1. Marcinakova and Laukova (2004) reported that *Enterococcus faecium* EF9296 produced a bacteriocin like substance against gram positive enterococci and *L. monocytogenes.* However, bacteriocins from LAB may also have inhibitory activity against other LAB (Settanni et al., 2005). Thus, it is likely that epiphytic microbes in silages may produce bacteriocins that could negatively affect the efficacy of microbial silage inoculants.

Silage inoculants must produce their desired end product(s) (e.g. lactic acid for classical homolactic lactic acid bacteria or acetic acid for *L. buchneri*) in sufficient quantities to affect specific epiphytic microbial populations. Ensuring that this happens in the silo may be very difficult at times. For example, although *S. bovis* HC5 produces a bacteriocin capable of inhibiting *Listeria*, the cell free bacteriocin probably is susceptible to breakdown by peptidases in silage (Contreras-Govea et al., 2008). Nsereko et al. (2008) developed a strain of *L. buchneri* capable of producing ferulic acid esterase which when used as a silage inoculant has the potential to improve fiber digestion in silages. However, digestion of NDF has not been consistently improved in several studies (Kang et al., 2009; Hofherr et al, 2008). Insufficient production of ferulic acid esterase under various conditions or instability of the enzyme in the silage mass could be a reason for this finding.

All microorganisms must also have sufficient amounts of water activity for growth. In heavily wilted and/or are extremely dry forages (>450 to 500 g DM kg^{-1}), silage fermentation is partially curtailed because of a lack of metabolic moisture for growth of lactobacilli. For example, in grass silage, only 10% of the total epiphytic LAB grew when the DM concentration was greater than 450 g kg^{-1} (Pahlow and Weissbach, 1996). Thus, liquid inoculants may be more advantageous since bacteria are added with their own moisture to hasten their rehydration and growth. Wardynski et al. (1993) reported that treating high moisture maize (about 700 g DM kg^{-1}) with a reconstituted liquid inoculant was more effective than using a dry inoculant in improving the subsequent fermentation. Addition of bacteria in water was more effective than a dry application of the same bacteria in lowering the pH of wilted (450 g DM kg^{-1}) grass silage (Pahlow and Weissbach, 1999) and wilted (550 g DM kg^{-1}) alfalfa silage (Whiter and Kung, 2001).

Targeting of the specific population of one type of detrimental epiphytic microbes makes the assumption that there are no other alternate routes for initiating the targeted effect. For example, addition of *L. buchneri* decreases the number of spoilage yeasts in silages because of moderate increases in acetic acid and therefore improves aerobic stability. However, aerobic spoilage could be initiated by *Acetobacter* rather than yeasts, as they would most likely be immune to the acetic acid. Failure of inoculants designed to improve aerobic stability via the inhibition of yeasts in some trials could be due to this reason.

Although a number of factors have been discussed that may explain why inoculants are not always effective, it is important to note that not all inoculants necessarily react the same to a given stressor. For example, the magnitude of the negative effect of chlorine differed depending on the organism tested (Muck, 1992). Similarly, we reported that the ability to withstand elevated temperatures in water varied depending on the specific inoculant tested (Mulrooney and Kung, 2008). The specific reasons for better thermotolerance in some of the bacteria than in others (in our study) are unknown but adaptation at moderately high temperatures (40 to 50°C) during growth has resulted in bacteria with more thermotolerance when they are heat shocked (55 to 65°C) (De Angelis et al., 2004). The ability of specific bacterial strains to withstand a multitude of stressors can be a key determinant for a successful inoculant.

Challenges Facing Other Silage Additives A variety of chemical additives have been added to silages. Additives such as sulfuric acid and unbuffered propionic acid are seldom used today because of their caustic nature. However, formic acid is still used in some areas. Chemicals such as buffered propionic acid, acetic acid, potassium sorbate and sodium benzoate have been added to silage to improve aerobic stability because they have good antifungal attributes. A major factor that could affect their efficacy on forage crops is pH because these acids are more effective when the pH is below their pKa values and they are mostly undissociated. Thus, high levels of these acids are required in high DM silage because lack of moisture limits the activity of LAB and the subsequent decline in pH. Another factor that could potentially affect the effectiveness of weak organic acids is the fact that several types of spoilage yeasts (e.g., *Issatchenkia orientalis* [*Candida krusei*] and *Pichia membranifaciens*) (Pitt and Hocking, 1997) and molds (e.g. Aspergillus niger [Plumridge et al., 2008] and *Penicillium* [Marth et al., 1996]) can be resistant to acid preservatives via degradation pathways (Casas et al., 2004) and expulsion mechanisms. In an unpublished study from our group (Neylon and Kung, 2000), the addition of buffered propionic acid was verified in freshly chopped maize but it was not detectable in the ensiled product suggesting that degradation of this acid is also possible during the ensiling process. Addition of propionic acid can also not overcome the fact that fermentable sugars are still needed in order for LAB to lower the pH and stabilize silages. Mills and Kung (2002) and Schmidt et al. (2004) both reported that propionic acid based additives could not overcome the effects of delayed filling (severe loss of fermentable sugars) and treated silages underwent clostridial fermentations.

Fibrolytic enzymes are a component of many silage additives. Inconsistent results from fibrolytic enzymes may be attributed to various factors. A negative interaction between fibrolytic enzymes and inoculant bacteria was observed by Stokes (1992). Enzyme application to mixed grass-legume silage at 280 to 300 g DM kg^{-1} improved silage fermentation, reduced forage fiber, and improved lactation performance but combination with a LAB inoculant negated enzyme

effects on fermentation and reduced the extent of fiber reduction. In contrast, inclusion of the same commercial LAB inoculant in a similar enzyme mixture had positive effects on silage fermentation of second and third cut alfalfa ensiled at 530 and 405 g DM kg^{-1} (Bolsen et al., 1989). Additionally, Cai and Ohmono (1995) reported that improvements in silage quality were ranked LAB + enzyme combination > LAB alone > enzymes alone when compared to untreated silage. A significant reduction of forage cell-wall fiber by an enzyme silage additive clearly requires the presence of the synergistic activities of multiple enzymes in the cellulase and hemicellulase enzyme complexes. Lack of, or lower levels of, one or more of the critical enzymes in the complexes could account for the poor effectiveness of some enzyme combinations in modifying silage composition or fermentation. Added enzymes could also be partially degraded by plant proteases that are released during chopping or inactivated by components in silage as Nsereko et al. (2000) reported that silage extracts partially inhibited a variety of exogenous enzymes.

Environmental factors may also affect the efficacy of enzymes as silage additives. If enzymes release fermentable substrate for use by LAB, they must be active early in the ensiling process at higher pH (5.5 to 6.4) and at lower temperatures than will occur later. These conditions may be considerably different from the enzyme's pH and temperature optima (pH 4.5 and 50°C for fungal cellulases). Enzymes also require binding with their substrates. Such binding is temperature related and could be an important factor in application of silage enzyme additives to crops harvested later in the season, such as maize silage, particularly in northern latitudes. The temperature optima of fibrolytic enzymes from *T. reesei* are approximately 50°C and activity was reduced by more than 70% at 26°C and more than 90% at 10°C (Sheperd and Kung, 1996). Few studies have actually tested the effect of temperature on the efficacy of enzymes on silage fermentation. The fermentation pattern of clover and ryegrass silages treated with both formic acid and a cellulase from *T. viride* (Henderson et al., 1982) was not affected by fermentation temperature (0, 15, 35, or 50°C). Wilting (Jacobs et al., 1991; Kung et al., 1991) may cause a reduction in enzyme activity because of reduced water activity at higher DM. Beuvink and Spoelstra (1994) reported that wilting up to 300 g DM kg^{-1} had no effect on gas production from grass silage treated with fibrolytic enzymes when compared to untreated silage, but wilting up to 488 g DM kg^{-1} increased the initial lag phase and lowered the rate and extent of gas production in treated silage. In addition, advancing maturity has reduced the effect of enzymes on silage fermentation and or degradation of fiber (Adogla-Bessa and Owen, 1995; Nadaeu et al., 2000; Sheperd and Kung, 1996) possibly because increased lignification of the cell wall makes it more resistant to attack by enzymes. Increasing the surface area of the forage for attack may increase enzyme accessibility to the substrate. Application of a cellulase preparation from *T. viride* to grass, alfalfa, or clover which was coarsely chopped, finely chopped, or minced before ensiling showed that the enzyme reduced silage pH and cellulose and increased the gain in water soluble carbohydrate concentration more in finely ground forages (Henderson et al., 1982).

Calibration of Applicators and Distribution of Silage Additives Proper calibration of applicators and distribution of all additives onto the forage mass is a critical part of using a silage additive. This is especially true with the advent of low water volume applications for inoculants that may result in only 30 to 60 ml of liquid per tonne of forage. Distribution of an additive is probably less than optimal when the "shower" method (e.g., a forage wagon drives under a scale and a set amount of liquid is showered on the top of the load) or "manual spread" method (e.g., a

person dips a small can into the source of inoculant and attempts to spread this over a large load of forage or area of forage mass) is used. In the early 1980s misapplication commonly occurred with the use of anhydrous ammonia. Although inadvertent under-treatment of an additive is of primary concern because of lack of effectiveness, over-treatment is also an issue because of cost and the potential of over treatment to have detrimental effects depending on the additive. I can remember specific maize silage samples that were supposed to have been treated with anhydrous ammonia but the chemical analysis showed almost zero recovery of added nitrogen primarily because of poor placement of the actual treatment. In contrast I can also remember specific analyses showing maize samples treated with anhydrous ammonia with nitrogen contents over 2.7% N (DM basis) because of over treatment. Extremely high rates of ammonia raised the pH of the forage mass to very high levels and resulted in prolonged fermentations that where often very high in acetic acid. Lastly, purposely applying less than the recommended rates of application to save money is a dubious practice because it only increases the probability that the additive will not be successful.

Interactions with Other Areas of Silage Management As noted earlier, the success of any silage additive could certainly be affected by harvest, storage, and feeding conditions. For example at harvest, slow packing rates, poor packing densities, delayed fills and inadequate sealing of silos could all potentially affect the efficacy of a silage additive. Challenges during silo filling could be detrimental to an inoculant because the upper layers of forage will remain poorly packed during the evening hours if filling is stopped. This can result in excessive respiration and high temperatures in the forage mass in those layers. During storage there is an increased chance of damage to plastic coverings with prolonged storage from a variety of issues such as weather and critter related damage. Even the best silage additives would have a difficult challenge when used in a bunker or pile silo that was never covered with plastic. During silage feed out low removal rates of silage, disruption of the feeding faces that allows air to penetrate into the silage mass, and extremely hot weather will challenge any silage additive. Faces of bunker, pile and bag silos will be more challenged during the hot summer months if they face the afternoon sun.

Conclusions When used correctly, various additives can help farmers maintain and sometimes improve the quality of their silages. However, a variety of factors can interact and affect the efficacy of a silage additive. Additives must be stored and applied properly to maximize their potential effectiveness. End users must realize that silage additives are not an alternative to good silage management practices.

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