Chapter 1

The Use of Probiotic Strains as Silage Inoculants

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Additional information is available at the end of the chapter

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1. Introduction

To secure the health and good performance of animal husbandry, animals need a constant supply of high quality nutrients the whole year round. The preservation of feed for use during periods of underproduction is a universal problem. All farmers worldwide face the challenge of guaranteeing feed for their animals throughout the year, and not only in terms of quantity but also quality [1, 2].

Thus, a major concern of any farm that seeks to operate economically is the need to preserve the quality of feedstuffs. On-farm feed preservation plays an important role in maintaining the nutritive value of feed while avoiding losses caused by micro-organisms and contamination with undesirable toxins, for instance, mycotoxins. Grain prices have risen steadily due to poor harvests in key producing countries, supply constraints in rice-growing economies and fast-growing demand for bio-fuel [3]. A price decrease is not expected in the coming years. This is one of the reasons why producers have to maximise animal performance by using locally produced feedstuffs that are found in abundance, such as pastures, silages and industrial by-products.

The preservation of feed value is an important topic for animal performance. The aim is to inhibit the growth of undesirable micro-organisms and the spoilage of the feedstuffs while minimizing nutrient and energy losses.

A common technique used to preserve feed involves manipulating the presence or lack of oxygen. Grains and hay are usually preserved aerobically with the addition of different preservatives. Ensiling is a classic example of an anaerobic preservation technique.

The practice of ensiling was originally a management tool used mainly in ruminant production to fulfill feed demand by storing and preserving any excess feed resources from periods of overproduction for later use during periods of lack. However, its importance has been increasing, especially in high input "zero-grazing" systems that enhance productivity

per animal per area unit [4-6]. Today, silage is the world's largest fermentation process, with an estimated 287 million tons produced in the EU alone [2].

Ensiling is a process in which lactic acid bacteria (LAB) convert sugars into mainly lactic acid and other by-products, such as acetic or butyric acid [7], under anaerobic conditions. This decreases the pH value, keeps the feed value, inhibits the growth of undesirable micro-organisms, and preserves forages for long periods of time under normal conditions of up to one to two years and even more. Though ensiling is used mainly to preserve voluminous feed, many other substrates including grains, by-products like fish residues, wet distillery grains with solubles or WDGS and brewer´s grains can also be ensiled.

The major advantages of silage are:

a. that crops can be harvested almost independent of weather conditions,
b. harvesting losses are reduced and more nutrients per area are harvested, and
c. ensiling permits the use of a wide range of crops [8, 9].

The necessary pre-requisites for the ensiling of any material are:

a. easily fermentable sugars (Water Soluble Carbohydrates, WSC),
b. anaerobic conditions,
c. lactic acid bacteria (LAB) and
d. factors allowing their proliferation like dry matter (DM) content and buffer capacity.

The DM content plays a huge role in the fermentability of a substrate. This key point seems to be easy to guarantee but under practical conditions, is actually not. Due to different weather conditions, it is a real challenge to harvest crops with adequate DM content.

On the other hand, bacteria, and specifically lactic acid bacteria originating from the epiphytic microflora or silage inoculants, are able to survive only under specific conditions. One such condition is the DM content, as it determines the osmotic pressure and the aw-value of the substrates.

The ensiling process can be divided into four main phases:

1. Aerobic phase: This refers to the respiration and proteolysis by the plant's own enzymes. This can be reduced by optimizing particle length and proper compacting of the material (Picture 1). This phase takes about three days under normal ensiling conditions.
2. Fermentation: This refers to the acidification caused mainly by lactic acid produced by lactic acid bacteria (LAB). This phase takes two to three weeks. Under anaerobic conditions, lactic acid bacteria produce considerable amounts of lactic acid and the pH decreases, inhibiting the growth of undesirable micro-organisms (especially *Clostridia* and *Enterobacteria*). LAB ferments the substrate homofermentatively (only lactic acid) or heterofermentatively (lactic acid + acetic acid). However, LAB represent only between 0.1 to 1.0 % of the normal epiphytic microflora. Therefore the use of bacterial inoculants to secure the fermentation has increased in recent years.

Picture 1. Compacting of corn whole plant for silage in a South African farm (Y. Acosta Aragón)

3. Stable phase: Fermentation ceases due to a lack of carbohydrate substrates, and the pH remains constant, depending on the anaerobic conditions created.
4. Feed out phase: Once the silo is opened and during feeding, portions of the silage are exposed to oxygen (Picture 2). Aerobic micro-organisms, primarily yeasts and molds, will grow, consume dry matter (sugar, lactic acid and other chemical substances), and cause heating and high losses (CO_2 and H_2O). This phase is decisive because the nutrient losses could be considerably high. Aliphatic short chain acids (acetic, propionic and butyric acid) [10] inhibit the growth of yeasts and molds and that is why biological inoculants containing heterofermentative bacteria are used. The response to additives depends not only on the forage to be treated, but also the dry matter (DM) content [11], sugar content, and buffering capacity of the original material [12]. The characteristics of inoculants include a rapid growth rate (to compete with other micro-organisms), tolerance of low pH, ability to reduce pH quickly, non-reactivity towards organic acids, tolerance towards a wide temperature range, ability to grow in high DM materials, absence of proteolytic activity and an ability to hydrolyze starch.

In recent years, producers have begun to pay more attention to silage additives, [13] which have been the focus of a tremendous amount of research over the last 20 years. Some of this research has focused on increasing the nutritional value of silage by improving fermentation

so that storage losses are reduced, and increasing the aerobic stability of silage after the opening of silos [14].

Picture 2. Silage after the opening of the silo under Brazilian conditions (Y. Acosta Aragón)

2. Silage microbiology

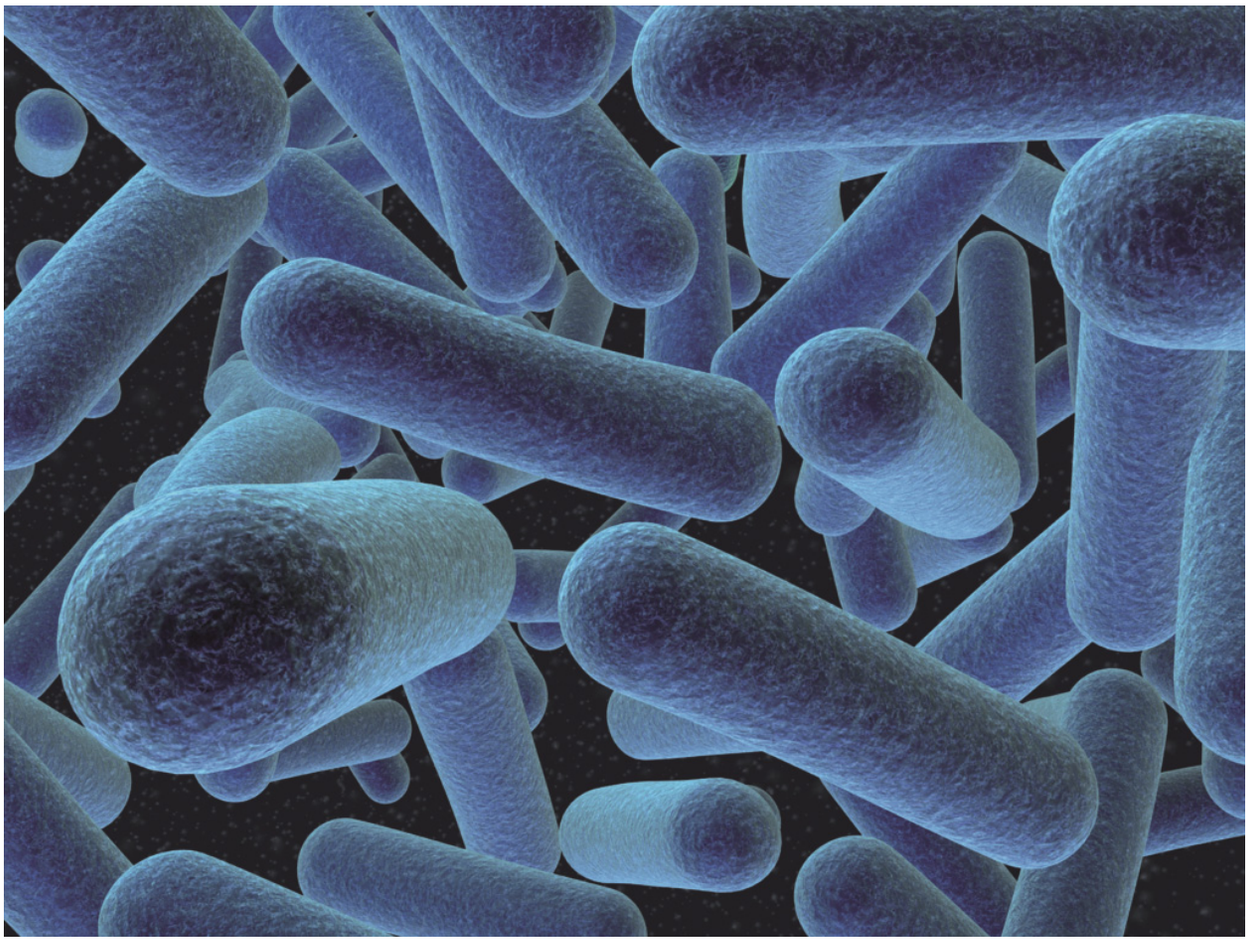
Silage making is based on microbiology. Silage inoculants are additives containing LAB that are used to manipulate and enhance fermentation in silages like grass, alfalfa, clover and other silages, as well as aerobic stability (mainly in corn silage). The most common LAB in commercial inoculants is *Lactobacillus plantarum* and other *Lactobacilli*, followed by *Enterococci* (for instance, *E. faecium*) and some *Pediococci* [15]. The main criteria for their selection are:

- high production of lactic and/ or acetic acid
- above all, quick growth in the first phase of the ensiling process in order to inhibit undesirable micro-organisms
- high osmotolerance
- fermentation under technical conditions
- no antibiotic resistances

One the most important classifications of the LAB is according to whether their influence on the ensiling process is homo- or heterofermentative. Homofermentative LAB produce

mainly lactic acid (more than 90% of the whole fermentation products) with energy losses close to zero. On the other hand, heterofermentative LAB use WSC not only to produce lactic acid but also acetic or propionic acid, ethanol, mannitol, etc.

The philosophy behind the first silage inoculants at the end of the 80s was that, in order to achieve good results in the ensiling process, the substrate needs to acidify very deeply and quickly. Since the drop in pH value is highly correlated (r^2 from -0.8 to -0.9) with the lactic acid content, a major goal was to increase the amount of lactic acid through the use of homofermentative LAB. However producers and researchers very soon found that the best fermented silages often showed a worsened aerobic stability after the opening of the silo. Those aerobic instabilities, reflected in heating and energy losses, are caused mainly by yeasts. Yeasts are aerobic, mostly unicellular, eukaryotic micro-organisms classified as fungi, which convert carbohydrates to CO_2 and alcohols, mainly ethanol. It is a metabolic exothermic process with an energy loss of approx. 40 %. However, yeasts are sensitive to short-chain organic acids like acetic and propionic acids. This was the reason for the start of the use of heterofermentative LAB to prevent aerobic silage instability.



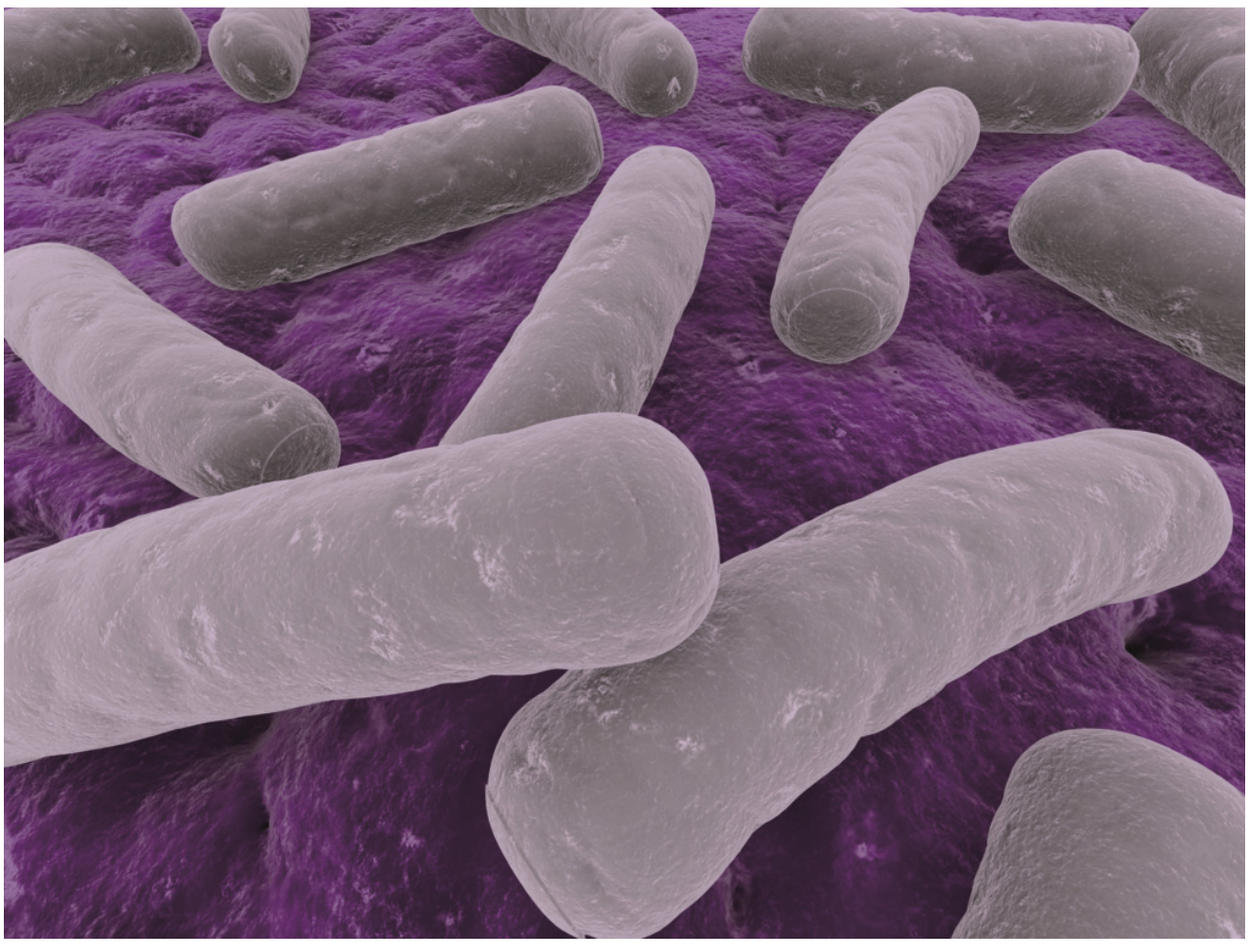
Picture 3. Listeria monocytogenes (iStock_000002507254Large©Sebastian Kaulitzki)

The main harmful micro-organisms present in silages are microbes with different characteristics (classification, physiology, pathogenesis, detection, epidemiology, routes of

infection, infectious cycles, etc.) [16]. Good agricultural practices can help to prevent infections transmitted by the ingestion of contaminated silages.

Listeria monocytogenes: These are gram-positive bacterium that can move within eukaryotic cells (Picture 3). Clinical symptoms, such as meningoencephalitis, abortions and mastitis in ruminants, are frequently recognized by veterinarians. The bacterium lives in the soil and in poorly made silage, and is acquired by ingestion. It is not contagious; over the course of a 30-year observation period of sheep disease in Morocco, the disease only appeared in the late 2000s when ensiled feed-corn bags became common. In Iceland, the disease is called silage sickness [17]. *L. monocytogenes* usually cannot survive below pH 5.6, but in poorly consolidated silage with some oxygen, it may survive at pH levels as low as 3.8. As these conditions also favor the growth of certain molds, moldy silage generally presents a high risk of listeriosis [18].

Clostridia: These are gram-positive obligate anaerobic bacterium that can form spores (Picture 4).

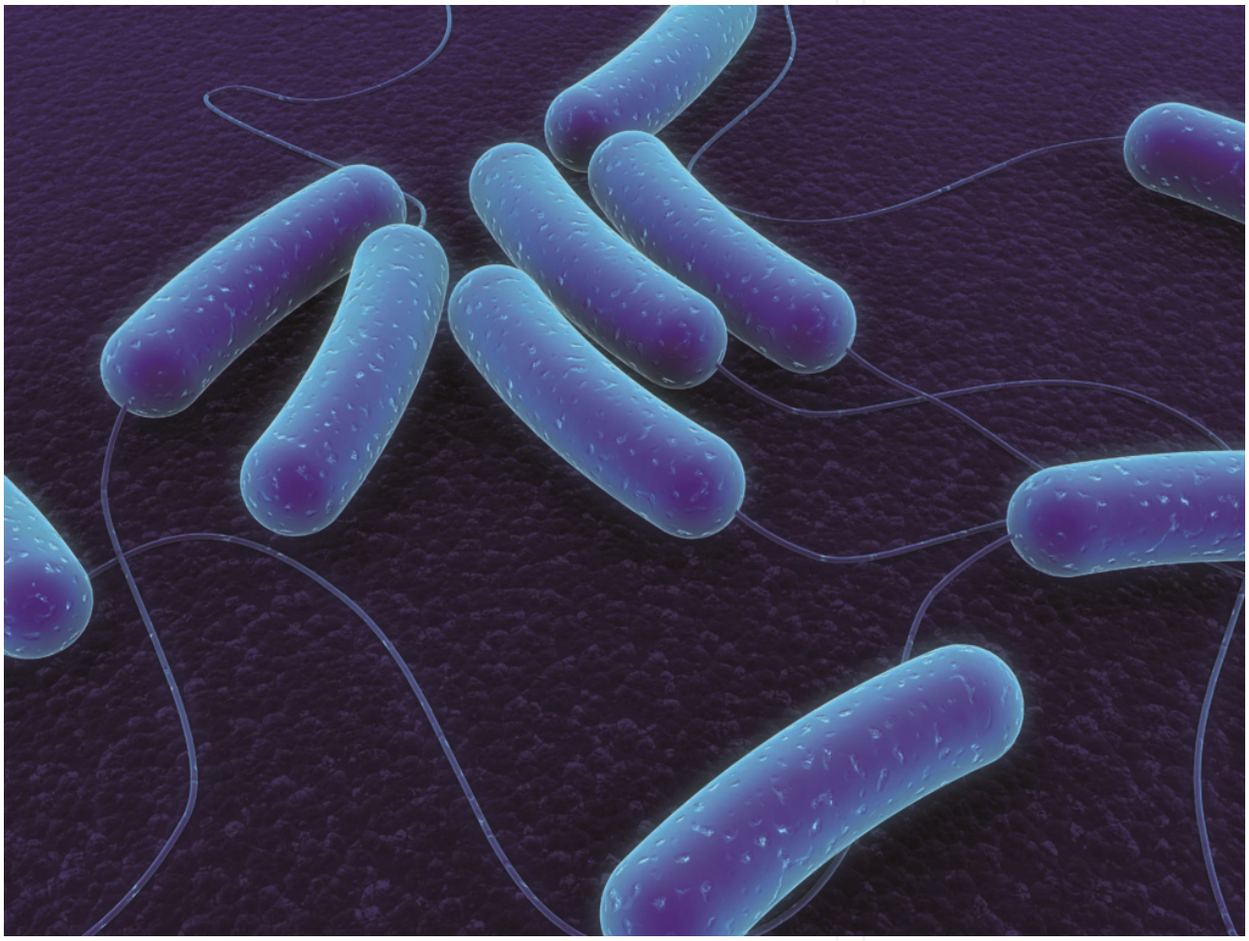


Picture 4. Clostridia (iStock_000008522722XLarge©Sebastian Kaulitzki)

Crops for ensiling are often harvested in relatively wet conditions and have a low dry matter content (<25 %). This presents a risk of contamination with Clostridia, which

increases the nutrient (protein) losses in silages and causes fermentation to butyric acid. Another important consequence is that animals may reject silage due to its low palatability. Clostridia can be prevented by a rapid and sudden pH decrease (pH below 4.5) [19].

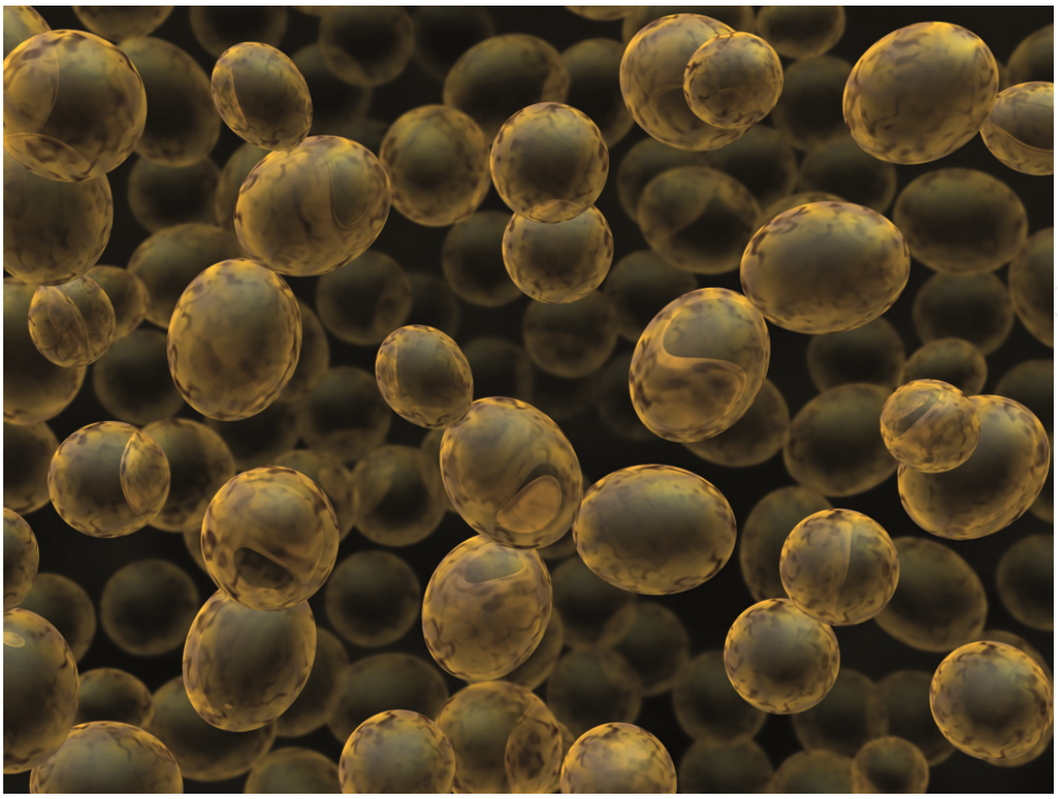
Entereobacteria (coli forms): These are gram-negative, non-spore forming, facultative anaerobes (Picture 5). They commonly enter silages from slurry, manure and soil in the early stages of fermentation and convert the water-soluble carbohydrates into acetic acid, ethanol, CO_2, and ammonia, resulting in high energy losses [20]. Their growth is reduced by anaerobiosis, low pH values and fermentation acids. The optimal pH value for growth is around 7; lower pH values markedly decrease the growth [20].



Picture 5. Enterobacteria (iStock_000003187348XLarge©Sebastian Kaulitzki)

Yeasts: These are eukaryotic unicellular aerobic micro-organisms (fungi) that use organic compounds as a source of energy, mostly from hexoses and disaccharides, and do not require sunlight to grow (Picture 6).

There are no known yeast species that only grow anaerobically (obligate anaerobes) [21]. Yeasts grow best in a neutral or slightly acidic pH environment. During the feed-out phase in the absence of inhibiting substances like acetic and propionic acid, yeasts can grow very rapidly and surpass 1 000 000 cfu/g silage, causing aerobic instability but also increasing the



Picture 6. Yeasts (iStock_000012250997XLarge©Dmitry Knorre)

Micro-organisms	Author	Year	Statement
Saccharomyces rouxii* and *Torulopsis versatilis	**Noda *et al.*** [23]	**1982**	An increased toxic effect in brine fermentation of soy sauce from pH 5.5 to 3.5
Candida krusei* and *Pichia subpelliculosa	**Danner *et al.*** [24]	**2003**	Acetic acid has the greatest inhibitory effect on yeast growth. 20 g liter^{-1} of acetic acid in the test mixture was enough to completely inhibit the growth of the selected yeasts at pH 4.
Silage yeasts	**Driehuis and van Wikselaar** [25] **Oude Elferink *et al.*** [18]	**1996** **1999**	High levels of formic or acetic acid reduce survival during storage (in silages)
Silage yeasts	**Driehuis *et al.* [26]** **Oude Elferink *et al.*** [18]	**1999** **1999**	Lactic acid is degraded anaerobically to acetic acid and 1,2-propanediol, which in turn causes a significant reduction in yeast numbers

Table 1. Effect of acetic acid on different yeasts

risk of diarrhea in domestic animals. They compete with lactic acid bacteria for sugars, which they ferment to create mainly ethanol. Ethanol has little (if any) preservative effect in the silage but causes extremely dry matter and high energy losses of 48.9 and 0.2 %

respectively [20]. A level of acetic acid of 1.5 to 3.0 % in the dry matter could prohibit yeast growth in silages exposed to air in the feed out phase [22]. However, higher levels diminish the silage palatability. An overview of results in the scientific literature about inhibition of yeast by acetic acid is presented in Table 1.

Molds*:* These grow in multicellular filaments and derive energy from the organic matter in which they live, for example silages (Picture 7).

Picture 7. Molds in silages (Y. Acosta Aragón)

Mold spores can remain airborne indefinitely, live for a long time, cling to clothing or fur, and survive extremes of temperature and pressure. Many molds also secrete mycotoxins which, together with hydrolytic enzymes, inhibit the growth of competing micro-organisms. The mycotoxins secreted can negatively affect the performance of domestic animals. Milk contamination, decreased milk production, mastitis, laminitis, poor reproductive performance and several gastrointestinal disorders are some of the effects on dairy cattle which have been extensively described. The main mycotoxins found in silages were ZON, DON and fumonisins [27] as well as roquefortine. The majority of fungi are strict aerobes (require oxygen to grow) [28]; and only a few of them are micro aerobic (*Mucor spp.)* [29]. The main parameters for controlling the growth of the micro-organisms as described above are summarized in *Table 2*.

Parameter	Micro-organisms				
	Listeria monocytogenes	*Clostridia*	*Enterobacteriae*	Yeasts	Molds
Nutrients (Water-soluble carbohydrates)	+++	+++	+++	+++	-
Anaerobiosis	+++	-	+++	+++	+++
pH*	+++	+++	+++	-	-
Lactic acid* (fermentation)	+++	+++	+++	-	-
Acetic acid* (feed out phase)	+	+	++	+++	+++

Table 2. The control of harmful micro-organisms present in silages
*- Low inhibition, + High inhibition. * Factors influenced by the use of silage inoculants*

3. Use of probiotic strains in silages

Fermentation characteristics are generally improved with inoculation [30]. [31] reported that inoculation improved fermentation characteristics in over 90% of 300 silages, including alfalfa, wheat, corn, and forage sorghum silages. With any forage preservation technique, the quantity and quality of material available at the end of storage is always below that of the original. Thus, the primary goal of forage preservation is to minimize the spoilage and losses of dry matter (DM) which will be reflected in the energy content of the silage, a limiting factor for milk production.

Silage inoculants can be classified according to their effect on the ensiled matter or their mode of action. The main effects of inoculants are:

a. to prevent undesirable fermentations and
b. to prevent silage spoilage during the feed out phase.

To achieve these effects, producers can utilize three different products or a combination of:

a. acids,
b. their salts and solutions respectively, and
c. biological silage inoculants.

Other silage additives with more limited uses than the above are molasses [32] and enzymes. Salts and acids are used to cause an abrupt decrease in the pH value when the dry matter content of the raw material is out of the optimal range. In cases of low dry matter content, these products inhibit, above all, the growth of *Clostridia*. High dry matter content very often means bad conditions for the compaction of raw materials; air stays inside the ensiled matter, thereby hindering the anaerobic conditions required for good silage. The advantage of the use of salts is that they are non-corrosive and easier and safer in application compared with their corresponding acids.

Biological silage inoculants have been used and are established on the market because of:

a. their proven effectiveness in accelerating fermentation and improving aerobic stability,
b. higher recovery of dry matter and energy content compared with non-treated silages,
c. safety during usage and
d. relatively lower cost per treated ton compared with acids.

The quality of good biological silage inoculants must be selected, first, on the basis of the included strains and their proportions in the product. Multi-strain inoculants have the advantage of possibly using different sources of energy, with each strain having a different desirable effect (rapid pH decrease, higher production of lactic acid, or acetic acid production for a better aerobic stability). It is, therefore, possible to change the mode of action of a product containing the same strains but with different proportions of the bacterial strains. On the other hand, different strains of the same micro-organism will grow faster on different substrates, temperature conditions or moisture content (osmotolerance).

Another aspect to take into account is the number of bacteria in the product and per gram of silage. A review of the products existing on the silage additive market shows a variation of 100 000 to 1 000 000 cfu/ g of silage [33].

The effectiveness of a biological silage additive can be measured using different methods. It is very difficult, under practical conditions, to measure success in terms of higher performance (milk and/ or meat production) because the whole process is conditional upon many factors. The first aspect to be taken into account is silage quality, worded in simple parameters such as pH value, fermentation acids and energy content, compared with the normal values for the ensiled crop or against a negative (no additive) or a positive (with other additive) control.

In selecting the right biological silage additive, some pre-requisites, such as the crop to be ensiled, should be taken into account. According to [33] there are three types of crops from the point of view of "ensilability", which are classified according to their fermentability coefficient (FC):

FC = DM + 8 x (sugar content / puffer capacity)

The following criteria are used to interpret the FC values:

- poor ensilability (FC < 35)
- average ensilability(35 < FC < 45) and
- good ensilability (FC > 35)

For substrates of poor ensilability, the recommended biological silage additive should contain (principally) homofermentative bacteria which produce mostly lactic acid. This dramatically reduces the pH value (high negative correlation coefficient of more than 0.80 between lactic acid content and pH values). For substrates of good ensilability such as in whole maize crop, the aim should be to increase the aerobic stability, because such substrates are very rich in nutrients and spoil very quickly when in contact with air, and

therefore yeasts and molds [26, 34]. In the last case (improvement of aerobic stability), biological silage additives with a higher ratio of heterofermentative bacteria are preferred due to a higher production of acetic or propionic acid and the corresponding inhibition of undesirable spoilage micro-organisms [35, 36]. Nevertheless the use of propionate-producing propionic bacteria appears to be less suitable for the improvement of silage aerobic stability, due to the fact that these bacteria are only able to proliferate and produce propionate if the silage pH remains relatively high [37].

A real challenge for probiotic strains is the inoculation of haylage because of the high DM content and the concomitant higher osmotic pressure. Very often, the term haylage is used indistinctly and there are definitions which claim that "a round bale silage (a baleage) is also sometimes called haylage". [38] considered baleage, big bale haylage and round bale silage as different names given to the same preserved feedstuff. Both processes are anaerobic but the first one (haylage) is related to the DM content at ensiling; and the second one (baleage) is the procedure used to protect the material against spoiling (baling, wrapping). That is the reason why we fully agree with [8] when he writes "wrapped haylage bales". Haylage may be preserved wrapped but also in other type of silos (bunker, trench, etc.). Another controversial topic is the right DM content range for haylage. A review on this topic is shown in Figure 1.

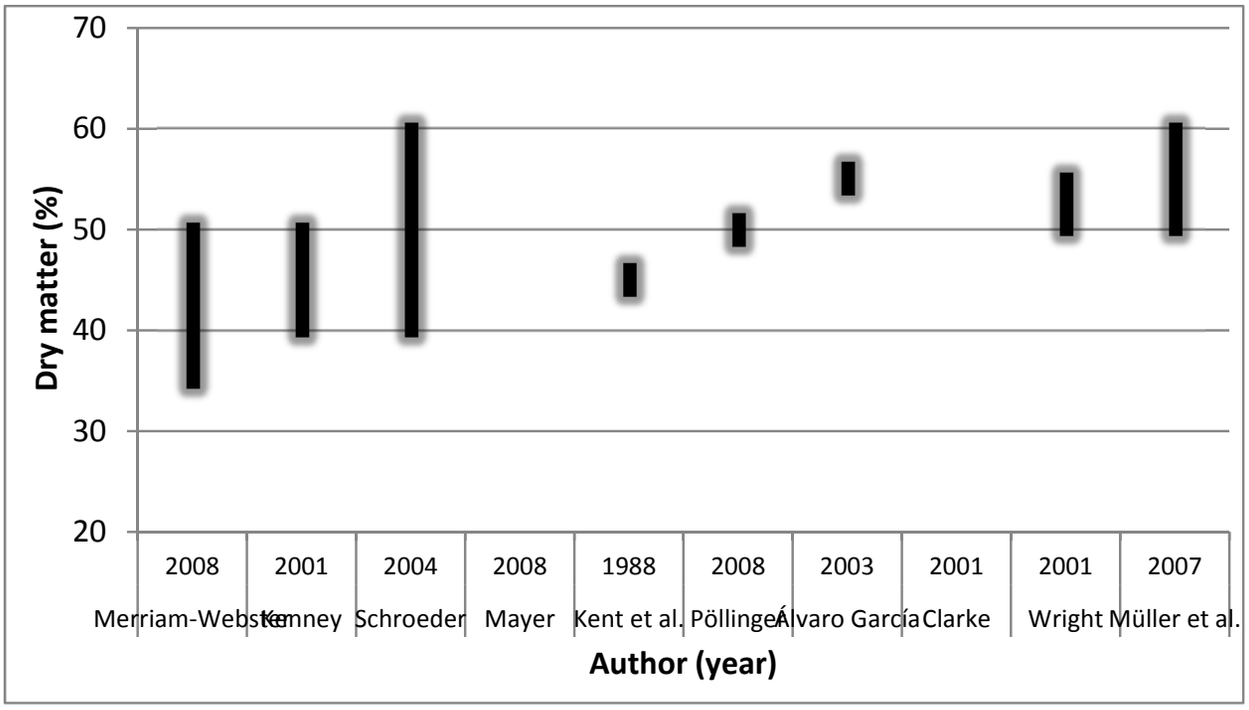


Figure 1. Dry matter content of haylage according to different sources

The range varies from 35 to 60 % DM. Moreover, many companies produce haylage for horses and consider it a special feed made of wilted grass silage with 65 % DM. In our context, where we refer to the use of silage inoculants in haylage for cattle, we will consider a range of 40 to 50 % DM, since anything below 40 % DM would be normal wilted silage. Anything over this range (55 % DM) and the feed would be more suited to horses due to the

higher fiber content *(see Figure 1).* Two very important aspects should be taken into account: a) the high DM content is out of the optimal values for LAB and b) the material, due to the high DM content, is difficult to compact.

The process of making haylage is the same as that for silage making, except that it takes longer for wilting to reach the desired DM content. The advantages of the use of haylage are:

- Free from spores and dust (very important for horses!)
- Lower storage losses than in silage making
- Weather independent compared with hay making
- Higher density of nutrients per volumetric unit compared with silages

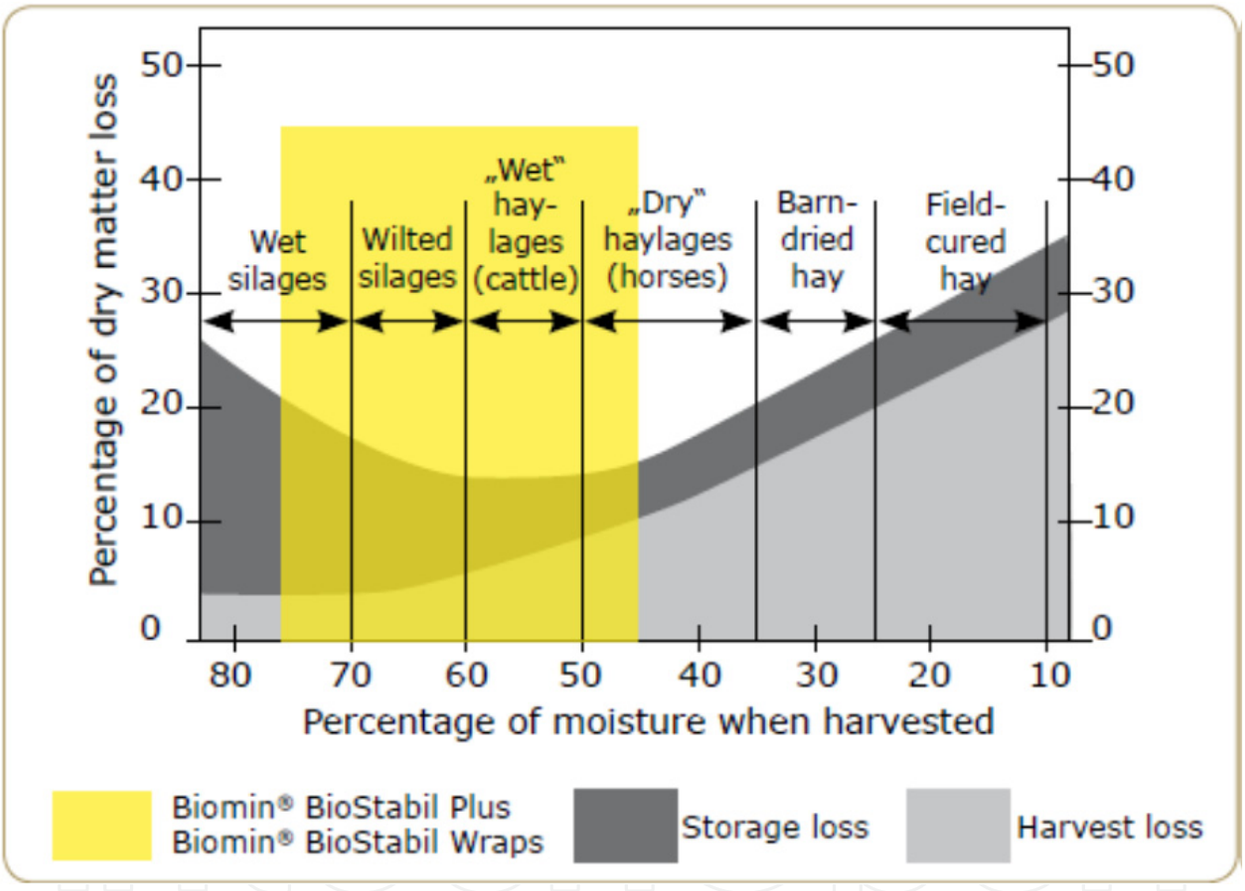


Figure 2. Estimated hay and haylage harvest and storage losses (adapted from [43])

The storage and harvest losses with different moisture contents are given in *Figure 2*. Note that total losses are minimized at a moisture level of between 50 and 60 % (40 to 50 % DM), which represents a great advantage of the use of haylage. According to [39], the quality parameters for haylage are not determined strictly enough. A major aim in haylage making should be to reduce pH values to below 5, ideally below 4.5 to diminish the risk of botulism [40] and listeriosis [41]. Since the DM is higher compared with that in silages, the production of fermentation products will be lower. Common values for haylage containing lactic and acetic acid would be from 15 to 50, and less than 20 g/ kg DM respectively. In haylage as in

silage, butyric acid and ethanol are equally undesirable. Due to the often slower acidification process, some amounts of one or both of these acidic substances may appear.

The effects silage inoculants in haylages should be the same as the effects in silages, namely. a quicker and deeper acidification and/ or enlarged aerobic stability, in addition to improved animal performance. [42] found a tendency towards higher DM intake (20.4 *vs*. 18.1 kg/ day) among cows in early lactation fed treated haylage (alfalfa haylage of 45 % DM; $P < 0.32$). The use of inoculants decreased the pH value from 5.29 *vs*. 5.11 for the control and the treated haylage groups respectively.

4. The control of harmful micro-organisms present in deficient silages

The examples are based on the results obtained in field trials with silages inoculated with blends of homo- and heterofermentative bacteria (Biomin® BioStabil Plus - 20 grass silages and Biomin® BioStabil Mays - 24 corn silages). Different substrates were used to refer to the silage quality parameters. In this study [44], only the parameters that can be directly influenced by the use of silage inoculants were selected (pH value, lactic and acetic acid and aerobic stability).

The results of the trials conducted with silages that have and have not been treated with silage inoculants are presented in Figure 3.

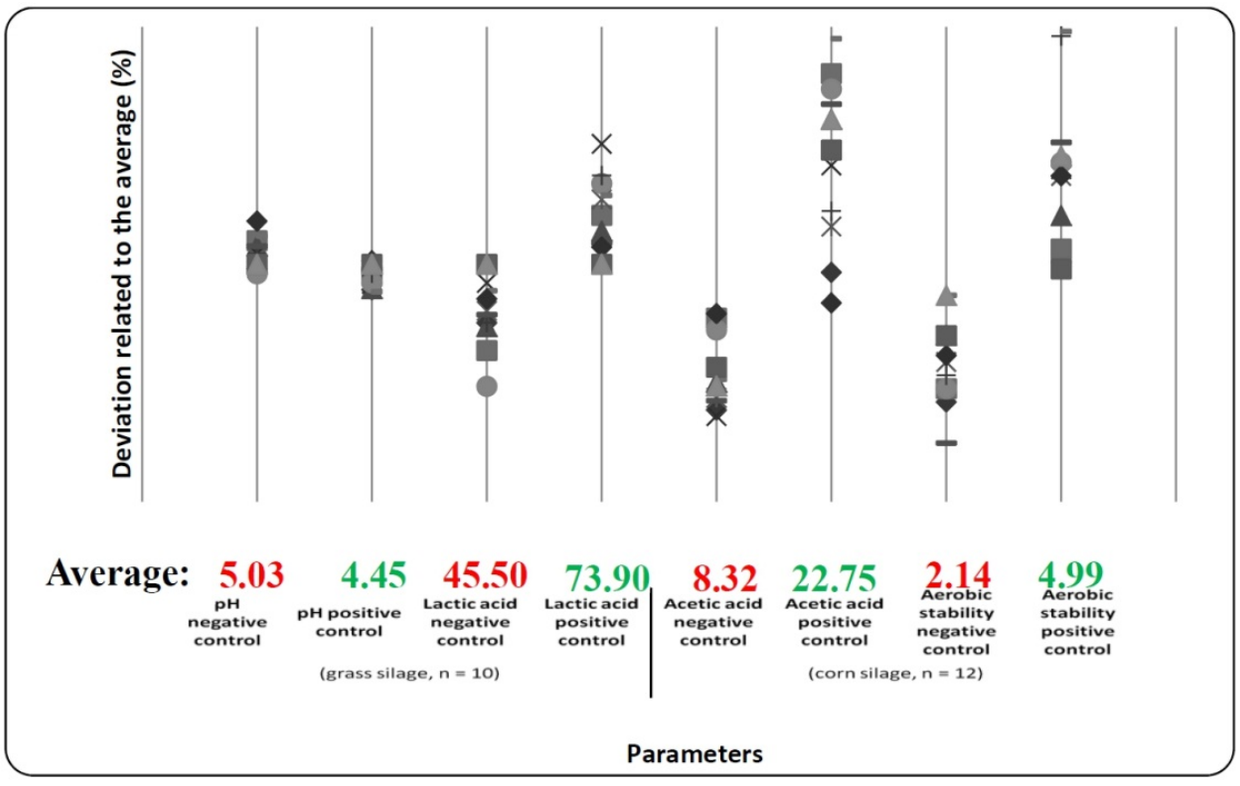


Figure 3. Influence of silage inoculants on selected parameters of the silage quality

As shown in Figure 3, the use of a silage inoculant improves the fermentation and lactic acid production (on average, 0.58 and in 28.4g/kg of dry matter respectively) in grass silages. The use of a silage inoculant that contains heterofermentative lactic acid bacteria (*L. brevis*) improves the acetic acid production and the aerobic stability in corn silages in 14.43g/kg of dry matter (+173 %) and 2.85 days (+133 %) respectively.

5. Results using probiotic strains in silages

The trial results were obtained with blends of homo- (*L. plantarum* and *E. faecium*) and heterofermentative bacteria (*L. brevis*) in different concentrations, as specified in each paragraph.

5.1. The use of silage inoculants in milk production

The use of silage inoculants can improve silage quality. Better silage means better hygiene and therefore improvements in animal performance can be expected. The results of a trial discussed below give an example of how milk production can be improved [45]. In the trial, mixed grass-legume sward wilted for 6 – 8 hours to 320 g DM/ kg (174 g of crude protein/ kg DM; 6.68 MJ NEL/ kg DM) was ensiled. The calculated fermentation coefficient was 49. The sward was cut and picked with a precision chop forage harvester (theoretical particle length of 30 mm). The grass-legume sward was treated with BSP (Biomin® BioStabil Plus, blend of *L. plantarum, E. faecium* and *L. brevis*; 2 x 10^5 cfu/ g of forage, 4 g of product applied in 4 liters of water/ ton), to be compared with a control treatment similarly collected from field but without inoculation after wilting. Representative samples of harvested and wilted grass mixtures were taken throughout harvesting. Silages were sampled every other week during the feeding experiment, which began 90 days after ensiling.

Aerobic stability was measured using data loggers which recorded the temperature once every six hours. The boxes were kept at a constant room temperature (21°C). Aerobic deterioration was denoted by the number of hours in which the temperature of the silage did not surpass the ambient temperature by more than 2°C.

Twenty-four Lithuanian black-and-white dairy cows were selected for the experiment from a larger group (from a herd of 120 dairy cows) according to parity, lactation, date of calving, present milk yield, last year's milk yield, and live weight using a multi-criteria method. The dairy cows were group-fed twice a day, bedded on straw and had access to water *ad libitum*. The cows were individually fed common commercial compound feed and their intake recorded.

Cows were milked twice a day and their milk yield was registered weekly. Milk samples were taken once a week from the morning and evening milking and the fat, protein, lactose contents and somatic cell count were analyzed. Data were analyzed using variance analysis to test for the effect of silage treatments with the software Genstat/ 1987. The Fisher's least significant difference (LSD) procedure at the 5% significance level was used to determine differences in treatment means.

There were no significant differences in the dry matter and crude fiber content (Table 3) between the untreated and treated silages. However, treatment with BSP resulted in significantly lower DM losses (+17.9 g/ kg of DM, P<0.01), significantly higher crude protein (149.4 *vs.* 159 g/ kg of DM; P<0.05) and digestible protein concentrations (108.9 *vs.* 117.8 g/ kg of DM; P<0.01). Kramer (2002) found higher dry matter losses due to fermentations that differed from the homofermentative and respirative processes in the ensiled material. Higher protein content was also found in silages treated with an inoculant by, for instance, [47] (legume grass mixture) and [48] (red clover). A quick reduction in the silage pH limits the breakdown of protein due to inactive plant proteases [49]. The net energy lactation (NEL) content was also significantly higher in the treatment with BSP (+0.08 MJ/ kg DM respectively).

Parameters	Unit	Treatments		P
		Control **X** ± SD	BSP **X** ± SD	
Dry matter (DM)	g/ kg	**315.4** ±3.12	**319.2** ±5.96	0.079
DM losses	g/ kg DM	**106.2** ±6.30	**88.3** ±6.75	**
Crude protein		**149.4** ±6.37	**159.0** ±6.91	*
Digestible protein		**108.9** ±5.92	**117.8** ±6.42	**
Crude ash		**70.7** ±5.04	**71.2** ±4.51	0.826
Net Energy Lactation (NEL)	MJ/ kg DM	**6.42** ±0.09	**6.50** ±0.07	*

Table 3. Effect of Biomin® BioStabil Plus treatment on the chemical composition of ensiled grass-legume

** and ** denote statistical significance at level 0.05 and 0.01 respectively*

The treatment with BSP increased fermentation rates, resulting in a significant pH decrease (P<0.05) and a significant increase in the concentration of total fermentation acids (P<0.05) compared with the control silage (Table 4). The inoculant produced more lactic acid (P<0.01), which reflects the results obtained by [50, 51, 52]; and numerically higher acetic acid content compared with that of the control silage. [6] gave a reference value of 1% for acetic acid in fresh matter to denote proper aerobic stability and good silage intake, whereas [53] gave a value of 2 – 3% in DM.

Both the butyric acid and ammonia nitrogen contents were significantly 10 times lower when BSP was used (P<0.01 in both cases). Butyric acid is the main product of the *Clostridia* metabolism, which can be controlled by a quick and deep acidification [46, 49]. [54] found no butyric acid in well fermented inoculated silages (pH of 4.1-4.2), while silages which

were not inoculated contained certain amounts of that acid. In more than 60% of reviewed literature, [52] reported lower ammonia nitrogen contents in silages treated with inoculants.

Parameters	Unit	Treatments		P
		Control X ± SD	BSP X ± SD	
pH	-	**4.38** ±0.09	**4.25** ±0.08	*
Total organic acids	g/ kg DM	**67.16** ±7.49	**76.62** ±8.60	*
Lactic acid		**36.74** ±5.26	**44.15** ±5.93	**
Acetic acid		**28.23** ±3.18	**32.17** ±5.43	0.051
Butyric acid		**2.15** ±1.98	**0.23** ±0.36	**
Ethanol		**7.87** ±1.16	**7.06** ±0.69	0.059
Ammonia N	g/ kg total N	**57.5** ±7.24	**46.0** ±4.03	**

Table 4. Effect of Biomin® BioStabil Plus treatment on the fermentation characteristics of ensiled grass-legume

** and ** denote statistical significance at level 0.05 and 0.01 respectively.*

The non-inoculated control silage was already heated after 54 hours and after 108 hours, had reached a temperature exceeding the ambient temperature by 2°C (Figure 4). The temperature rise in inoculated silage was small and first heated after 102 hours; however, no temperature rise of 2°C over the ambient temperature was observed during the 10-day exposure to air. This is due to a higher acetic acid content, which stops yeast growth. Increased concentrations of acetic acid in silage treated with BSP had a positive effect on the aerobic stability of the silage [24, 55].

Classical microbial inoculants, containing only homolactic bacteria, were shown to have no effect on and could even cause the aerobic stability of the silage to deteriorate [52, 56]. [57] found no positive effect on aerobic stability when a blend of homolactic lactic acid bacteria was used. Several authors have discovered that heterolactic lactic acid bacteria positively improve aerobic stability [24, 58].

Silages and dry matter intake are presented in Table 5. Based on the data recorded during the experimental period (92 days) the feed intake of silage DM was higher by 6.5% for treated silage than that of the untreated silage, corresponding to the results from [59]. The intake of compound feed did not differ as it was restricted to a certain amount for both treatments. The energy intake (digestible energy and net energy lactation) was also higher for the silage treated with BSP (+6.1 and 5.3 % respectively) compared with the untreated

control treatment. The Energy Corrected Milk (ECM) production was also higher in the BSP treatment (+1.4 liter of ECM/ cow/ day). [55] reported a milk production increase of 3 – 5%. [52] reported increased milk production in approx. 50% of the reviewed studies, with a statistically significant average improvement of +1.41 l/ day.

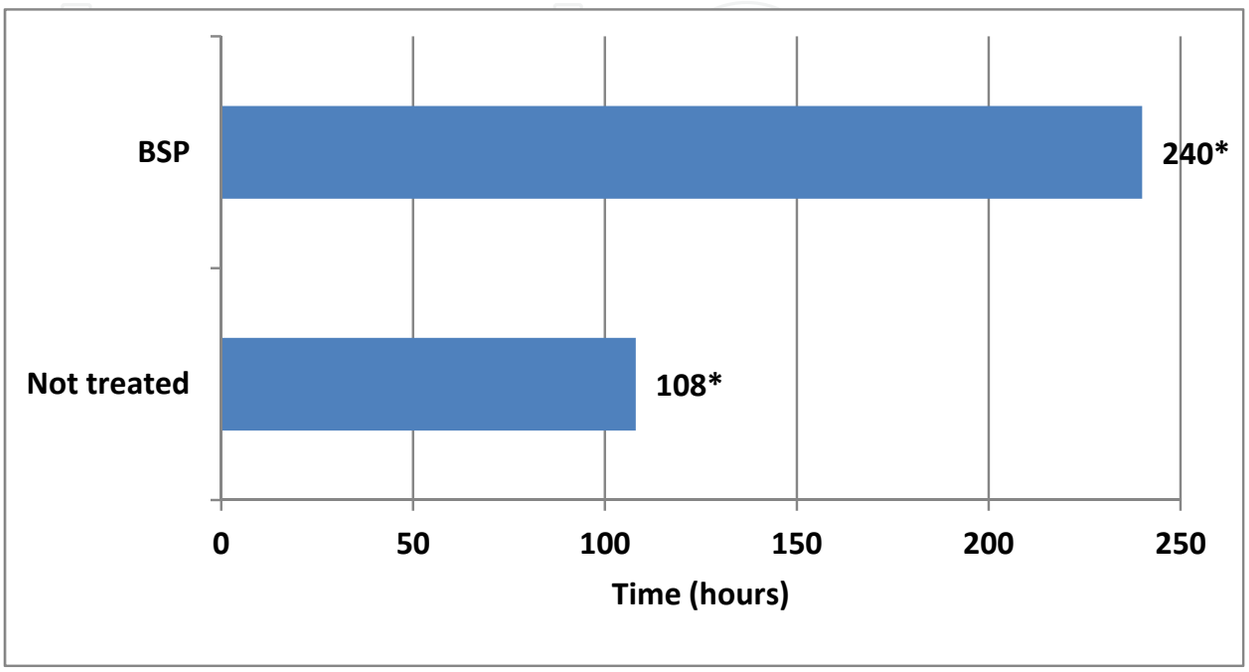


Figure 4. Aerobic stability of grass-legume silages treated or not with a silage inoculant
(and ** denote statistical significance of means at 0.05 and 0.01 levels respectively)*

Parameters	Unit	Treatments		P
		Control X ± SD	BSP X ± SD	
Silage intake	kg DM/ cow/ day	**10.7** ±1.51	**11.4** ±1.26	0.225
Compound feed		**4.0** ±0.61	**4.0** ±0.49	0.988
Total Dry matter intake		**14.7** ±2.12	**15.4** **±1.74**	0.382
Total Net energy lactation intake	MJ	**103.0** ±14.94	**108.5** ±12.33	0.341
Daily energy corrected milk (ECM) production	kg/ cow/ day	**17.4** ±2.69	**18.8** ±2.40	0.183
Feed Conversion (FC)	NEL MJ/ 1 kg ECM	**5.93** ±0.08	**5.77** ±0.09	**

Table 5. The effect of inoculant Biomin® BioStabil Plus on silage intake, milk yield and feed conversion
** and ** denote statistical significance at level 0.05 and 0.01 respectively.*

The feed conversion, calculated as the quotient between the NEL intake and the ECM production, denoted better efficiency in the conversion of energy into milk in the treatment with the BSP inoculant: cows fed the treated silage needed less energy (5.77 MJ NEL/ 1 liter of ECM) than others fed an untreated silage (5.93 MJ NEL/ 1 liter of ECM). This difference of 0.16 MJ was of high statistical significance (P<0.01), in spite of the fact that the differences in the parameters silage intake and milk production were not statistically significant. According to [55], feed efficiency can be increased by up to 9%.

The milk composition and somatic cell count are shown in Table 6. The protein, fat and lactose contents were higher in the BSP treatment, but not statistically significant (P>0.05). The somatic cell count of the milk from cows fed the treated silage was of statistically lower significance (P<0.05) than that of the control treatment (125,000 *vs*. 222,000). This correlates with improved hygiene in the treated silage. This parameter of milk quality should be considered as a consequential effect of better silage hygiene. It is well known that the somatic cell count is a polyfactorial parameter [60, 61].

Parameters	Unit	Treatments		P
		Control **X** ± SD	BSP **X** ± SD	
Fat	%	**4.30** ±0.40	4.43 ±0.28	0.376
Protein		**3.36** ±0.15	**3.42** ±0.22	0.451
Lactose		**4.80** ±0.15	**4.87** ±0.19	0.317
Somatic cell count	1000	**222.3** ±152.13	**125.1** ±30.98	*

Table 6. The effect of inoculant Biomin® BioStabil Plus on milk constituents and the somatic cell count
** and ** denote statistical significance at level 0.05 and 0.01 respectively.*

The biological silage inoculant had a significant effect on the quality characteristics of legume-grass silage, in terms of lower pH, due to a higher lactic acid fermentation caused by the homofermentative lactic acid bacteria. Similarly, inoculated silage showed higher (P<0.05) net energy lactation concentrations by 1.25%, compared with untreated silage. Inoculant treatment significantly decreased butyric acid content, N-NH_3 fraction and dry matter losses.

Improved silage fermentation with BSP increased silage intake and milk production. Better utilization of feed energy was reflected in the significantly higher efficiency of the conversion of feed-NEL into milk. Significantly lower somatic cell counts in milk from cows fed with the treated silage, indicate a higher hygiene quality in the milk compared with that of the control treatment.

5.2. The use of silage inoculants in meat production

The use of silage inoculants in the production of meat has been widely investigated [62, 63]. In spite of the sometimes controversial results, several trials have shown advantages from their use, reflected in better silage quality, aerobic stability and animal performance. The results of a trial conducted by [64] will be discussed in detail in the following paragraphs.

The aim of this trial was to study the effect of a silage inoculant on the nutrient content, silage quality, aerobic stability and nutritive value of ensiled whole plant corn, as well as on the feed intake and growth performance of fattening young cattle.

The effect of inoculation for whole plant corn silage treated with a commercial product (Biomin® BioStabil Mays, BSM, blend *Enterococcus faecium, Lactobacillus plantarum* and *Lactobacillus brevis*, DSM numbers 3530, 19457 and 23231 respectively; 4 g of product/ton of silage diluted in 4 l of water, 1 x 10^5 cfu/g of material), was compared with a control treatment with no silage additives (CT). The material had a DM of 323 g/kg, crude protein and water soluble carbohydrate concentrations of 87.9 and 110.5 g/kg DM respectively.

The inoculant was applied uniformly using an applicator. The silos were filled within 48 hours, covered with polythene sheet and weighted down with tires. The raw material as well as each silage was sampled. Volatile fatty acid and lactic acid, as well as alcohol concentrations, were determined by gas-liquid chromatography.

Aerobic stability was measured using data loggers which recorded temperature readings once every six hours. The boxes were kept at a constant room temperature of 21°C. Aerobic deterioration was denoted by days (or hours) until the start of a sustained increase in temperature by more than 2°C above the ambient temperature.

For the animal feeding trial 40 young beef cattle (eight to nine months old) with similar mean live weights were used and divided into two analogous groups (20 animals each). The experimental period lasted 100 days.

The animals were bedded on straw and had free access to water. Fresh silages were offered *ad libitum* twice daily, allowing for at least 10% orts (as-fed basis). Silage DM intake was calculated per group as the difference between the amount of silage supplied and the amount of silage remaining. Barley straw was included in the diet (1 kg/ animal/ day; 88 % of DM, energy value of 3.9 MJ ME/ kg DM). The animals were individually weighed on the first day of the experimental period, subsequently once per month, and on the final day of the experiment. The average weight gain and growth rates were calculated for each animal and for each group. Feed conversion ratio was calculated as the ratio between feed intake and body weight gain. Data were analyzed using variance analysis to test for the effect of silage treatments by Genstat/ 1987. A probability of $0.05<P<0.10$ was considered a near-significant trend.

The use of BSM significantly improved the silage quality compared with the CT (Table 7). The silage treated with BSM showed statistically significant higher DM recovery and digestible protein, coinciding with [65]; lower DM losses ($P<0.01$ for all) and higher crude

protein content (P<0.05). The digestible energy content was highly significant in the treated silage compared with the untreated silage. There were no significant differences between the untreated and treated silages in terms of crude fiber NDF content.

Parameters	Unit	Treatments		P
		Control X ±SD	BSM X ±SD	
Dry matter (DM)	g/ kg	**305.8** ±4.30	**312.2** ±4.66	**
DM losses	g/ kg DM	**70.2** ±15.87	**40.9** ±2.60	**
Crude protein		**80.2** ±4.94	**84.7** ±3.24	*
Digestible protein		**48.2** ±2.96	**52.5** ±2.01	**
Crude fiber		**214.8** ±4.59	**210.2** ±7.30	0.074
Crude ash		**45.2** ±3.26	**44.4** ±4.10	0.622
Digestible Energy (DE)	MJ/ kg DM	**12.8** ±0.06	**13.1** ±0.07	**
Metabolizable Energy (ME)		**10.8** ±0.08	**10.9** ±0.13	*

Table 7. Effect of the treatment with a commercial product BSM on the chemical composition and fermentation characteristics of ensiled whole plant corn
** and ** denote significance at level 0.05 and 0.01 respectively*

BSM treatment increased fermentation rates in whole crop corn silages, resulting in a significant pH decrease (P<0.01) and a significant increase in total organic acids concentration (P<0.05) compared with the CT (Table 8). The lactic acid content in the BSM treatment was also significantly higher (P<0.01) since homofermentative LAB were used [66]. The acetic acid content of the BSM treatment was numerically higher than that of the CT. Silage inoculation with BSM significantly decreased concentrations of butyric acid, ethanol and ammonia-N (P<0.01) of corn silage compared with the CT. Homofermentative silage inoculants by improving silage fermentation can reduce wasteful end-products such as ammonia-N and volatile fatty acids, which result in poorer feed conversion efficiency and higher in-silo dry matter losses [67-70].

The use of silage inoculants containing homofermentative lactic acid bacteria to increase lactic acid production and enhance the rate and extent of pH decline [12, 37, 70] can also lead to a reduction in protein breakdown [65]. As shown in Table 2, the BSM silage treatment decreased DM losses by 3.0 % (P<0.01) and had higher digestible energy (DE) and metabolic energy (ME) concentrations by 2.3 and 1.00 % (P<0.01 and P<0.05) respectively compared with the untreated CT silage.

Parameters	Unit	Treatments		P
		Control X ±SD	BSM X ±SD	
pH	-	**3.89** ±0.09	**3.71** ±0.03	**
Total organic acids	g/ kg DM	**80.0** ±4.33	**93.3** ±10.52	**
Lactic acid		**50.3** ±2.60	**61.4** ±5.88	**
Acetic acid		**29.0** ±2.16	**31.5** ±4.87	0.116
Butyric acid		**0.4** ±0.30	**0.1** ±0.11	**
Ethanol		**13.2** ±2.10	**9.3** ±2.41	**
Ammonia N	g/ kg total N	**51.0** ±10.29	**38.0** ±7.77	**

Table 8. Effect of the treatment with a commercial product BSM on the fermentation characteristics of ensiled corn
** and ** denote significance at level 0.05 and 0.01 respectively*

During aerobic exposure after opening the silos, the CT (Figure 5) had a temperature increase of more than 2°C above the ambient temperature after 84 hours. In the BSM treatment, the increase of more than 2°C above the ambient temperature occurred only after 156 hours.

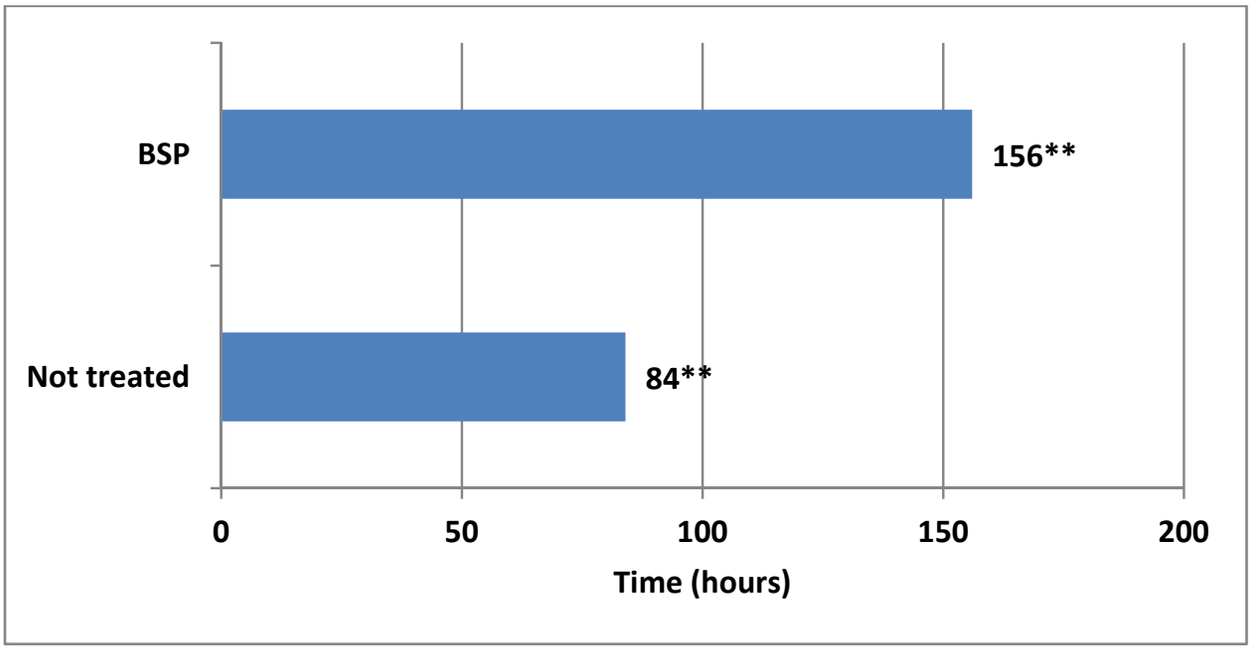


Figure 5. Aerobic stability of corn silages treated or not with a silage inoculant
(and ** denote statistical significance of means at 0.05 and 0.01 levels respectively)*

The stability of BSM silage was improved by 72 hours (3 days) compared with the CT. Recently, silage studies with whole crop corn silages using obligatory heterofermentative LAB *L. buchneri* as an inoculant, showed a 20-fold increase in the aerobic stability of the silage, which increased from approximately 40 hours for untreated silages to more than 790 hours for the inoculated silages [26]. Other studies [58, 71] have provided more definitive evidence of the existence of certain LAB strains with the power to inhibit yeast and mold growth, and to improve aerobic stability. Some authors have described the positive aspect of the formation of acetic acid by heterofermentative lactic acid bacteria, which inhibits spoilage organisms [7, 72].

Average daily weight gains (ADWG) for BSM and CT are shown in Table 9.

Treatment/ statistical parameter	**n**	**Trial period in days** (kg, **X** ±SD)			
		0 - 31	**32 - 63**	**64 - 100**	**0 - 100**
Control	20	**0.931** ±0.124	**0.981** ±0.129	**1.068** ±0.074	**0.998** ±0.087
Commercial product BSM	20	**0.940** ±0.081	**1.062** ±0.129	**1.206** ±0.089	**1.078** ±0.078
Standard error	-	0.016	0.021	0.017	0.014
P level	-	0.778	0.055	**	**

Table 9. Average daily body weight gain of the beef cattle in different trial periods
** *denotes significance at level 0.01*

From 0 to 31 trial days, neither statistically nor numerically marked differences in ADWG were found between the treatments. However in the trial period between 32 to 63 days, the differences in ADWG show a near-significant trend ($0.05<P<0.10$) with a P value of 0.055. The ADWG in the last third of the feeding trial period (from 64 to 100 days), and throughout the whole trial period (0 to 100 days), showed a statistically significant difference ($P<0.01$) of 138 and 80g respectively.

In order to avoid differences due to different moisture contents, the intake is shown in Table 10 on the DM basis. The silage DM intake for BSM was higher by 6.14% compared with the CT (3.97 *vs.* 3.74 kg DM/ animal/ day), and showed a near-significant trend ($P=0.065$). As expected, because of the restricted feeding, no differences were found in compound feed DM intake. These results were similar to those reported by [52]; however, some researchers found that feeding microbial inoculated silage to cattle does not affect dry matter intake compared with non-inoculated silage [73]. A combination of increased DM intake and higher energy in the silage treated with BSM, led to a significant increase ($P<0.05$) in metabolizable energy intake compared with those animals fed with the CT. The animals receiving BSM had a better conversion of energy into body weight compared with that of the CT because they needed 2.37 MJ of ME (3.4 %) less for a 1 kg increase in body weight. However, this difference was not statistically proven.

Parameter	Unit	Treatment		p
		Control X ±SD	BSM X ±SD	
Silage DM intake	kg DM/ animal/ day	**3.74** ±0.12	**3.97** ±0.17	0.065
Compound feed DM intake		**1.74** ±0.0	**1.74** ±0.0	0.000
Total DM intake[1]		**6.36** ±0.12	**6.59** ±0.17	0.065
Total Metabolizable Energy (ME) intake	MJ/ animal/ day	**69.27** ±1.33	**72.34** ±1.97	*
Feed Conversion Rate	MJ of ME / kg gain	**69.52** ±3.49	**67.15** ±2.26	0.298

Table 10. The effect of the treatment with the commercial product BSM on silage DM, energy intake, and feed conversion rate

** denotes statistical significance at level 0.05*

[1] 1 kg/ animal/ day of barley straw (88% of DM, 3.9 MJ ME/ kg DM) was included in the diet for both treatments

The inoculation with the microbial silage inoculant had a significant positive effect on whole crop corn silage quality in terms of:

- lowering pH and shifting fermentation towards lactic acid,
- suppressing butyric acid, ethanol and ammonia-N formation,
- significantly reducing DM losses,
- statistically increasing digestible and metabolizable energy,
- statistically significant improvements in aerobic stability, and
- improvements in the silage intake and performance of beef cattle, and a positive effect on the utilization of feed energy.

6. Limiting factors in the use of probiotic strains for silages on the farm

Many factors have been associated with failures in the use of probiotic strains as silage inoculants. They could be related to ambient factors, to the strains themselves and to the application.

6.1. Limiting factors related to the ambient

- **Water soluble carbohydrates (WSC)**: These are main sources of energy for lactic acid bacteria. There is a lack of WSC in crops wilted for long periods [74]. Low concentrations of WSC in herbage, even in inoculated ones, can lead to a decrease in silage quality [75, 76].
- **Water content and water activity in the crop**: The lack of water in the material to be ensiled can seriously affect the growth of LAB. Harvesting at low moisture levels worsens the compacting and therefore the exclusion of oxygen in the ensiled material.

- **Ambient temperatures at ensiling**: Extreme low or high temperatures can affect the performance of probiotic strains used as silage inoculants. Regions in Northern Europe and Canada could be affected by low temperatures in September/ October, in some cases below 0°C during the night. However it is important to note that daytime temperatures which coincide with the time of silage making are more important. Ambient temperatures of around 10°C during silage making could be considered the lowest limit for the activity of probiotic strains [77]. On the other hand, a combination of high temperatures (>35°C) and high humidity could negatively influence the ensiling process. It is well known that *Pediococci* are more resistant to higher temperatures than *Lactobacilli* [78], which could lead to the possibility of developing silage inoculants for tropical regions.

6.2. Limiting factors related to probiotic strains

- **Viability of the probiotic strains**: This is closely related to storage conditions. High temperatures and/ or high humidity have been associated with lower survival rates in available commercial products (DLG, 2011). The shelf life varies between six months (granulates) and 18 to 24 months (powders for liquid application).
- **Competitiveness vs. epiphytic microflora**: Bacteria contained in the silage inoculants have to compete successfully against the wild microflora living on plants. Many probiotic strains fail in the selection process for silage additives due to their low capacity to grow more rapidly or suppress other undesirable micro-organisms. A classic example is *Propionibacterium* where the production of propionic acid could be of great importance in extending the duration of silage aerobic stability. Unfortunately *Propionibacterium* grows more slowly than other bacteria and is affected by low pH values [79, 80].
- **Concentration of the probiotic strains in commercial products**: The scientific community [78] and manufacturers [33] agree that the minimal concentration of lactic acid bacteria is 1×10^5 cfu/ g of silage. The concentration in the silage can be easily calculated by multiplying the concentration in the product by the dosage per ton, and dividing by 1×10^6. As simple as this seems, big differences between declared concentrations and real concentrations have been found in our own research. However, the concentration of in cfu/ g of silage cannot be the only criterion for selecting a silage inoculant. Selection must also include the ability to decrease the pH value (high lactic acid production) and/ or improve the aerobic stability (for example acetic acid production).

6.3. Limiting factors related to the application

- **Quality of diluted water**: It is a well-researched fact that chlorinated water can decrease the effectiveness of probiotic strains. One important aspect is also the microbiological quality of water. Often, water is contaminated with *E. coli*, the bacterium responsible for nutrient losses and fecal odor in the silage.

- **Tank shelf life**: Storage conditions in the applicator tank differ in terms of temperature, chlorine content, toxic residues and sunlight. It is therefore strongly recommended that products are used within 24 to 48 hours after dilution. The user should be aware that he is working with live micro-organisms which can survive and be effective only if favorable conditions are created for them. An important selling point, for example, was in Australia where the tank shelf life was extended by over one week. Special attention should be paid to that: it is not about what is easier, but what is more effective.
- **Dry application vs. powder application**: Addition of bacteria to water was more effective than a dry application of the same bacteria in lowering the pH of wilted grass silage and wilted alfalfa silage (450 and 550 g DM/ kg) [81, 82, cited by 74].

Abbreviations

BSM	Biomin® BioStabil Mays
BSP	Biomin® BioStabil Plus
cfu	Colony forming units
CT	Control treatment
DE	Digestible energy
DM	Dry matter
ECM	Energy corrected milk
LAB	Lactic acid bacteria
ME	Metabolizable energy
NEL	Net energy lactation
WSC	Water soluble carbohydrates

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