Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Department of Animal Nutrition and Management

The effect of a feruloyl esterase producing inoculant along with mechanical treatment prior to ensiling on fibre digestibility of grass silage

Kiros Kelkay Haile

Department of Animal Nutrition and management
Degree project • 30 credits
Uppsala 2019

The effect of a feruloyl esterase producing inoculant along with mechanical treatment prior to ensiling on fibre digestibility of grass silage

Kiros Kelkay Haile

Supervisor: Kamyar Mogodiniyai Kasmaei, Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management

Examiner: Rolf Spörndly, Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management

Credits: 30 credits
Level: Second cycle, A2E
Course title: Degree Project in Animal Science
Course code: Ex0870
Course coordinating department: Department of Animal Nutrition and Management

Place of publication: Uppsala
Year of publication: 2019
Online publication: https://stud.epsilon.slu.se

Keywords: **ryegrass, meadow fescue, ensiling, *in vitro* incubation, feruloyl esterase, fibre digestibility.**

Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science
Department of Animal Nutrition and Management

Abstract

Plant fibre is the main portion of dairy cattle diets. It is also important in maintaining health and proper function of the rumen. However, digestibility of fibre is relatively low, and, in most cases, it is not more than 60%. In cell walls of grasses and cereals, lignin and hemicellulose are connected, forming a matrix. This matrix coats the cellulose and this overall structure of fibre is considered as the key reason for a low fibre digestibility. The main binding component between lignin and hemicellulose is ferulic acid. Feruloyl esterases (FAEs) are enzymes that can cleave the ferulic acid linkages between lignin and hemicellulose. The objective of the study was to investigate the effect of a FAE producing inoculant (FAEI) along with physical treatment prior to ensiling on digestibility of silage fibres. Ryegrass and meadow fescue samples were collected during autumn 2018, chopped and thereafter frozen (-20^{o}C) until the trial was started. At the time of the trial (2019), the grass samples were thawed and wilted in room temperature until the dry matter (DM) content reached ~35%. Six treatments, in triplicate, were compared for the effect on neutral detergent fibre digestibility (NDFD) of the silages. The treatments were untreated control, inoculation with *Lactobacillus buchneri* LN4017, Mild, Harsh, inoculation plus Mild and inoculation plus Harsh. The Mild treatment was pounding grass samples with a metal rod and the Harsh treatment was mincing grass samples with a meat mincer. After application of treatments, forages were ensiled in glass silos (100 mL) for 48-49 d. The NDFD of silage was assessed by a 96-h *in vitro* incubation with buffered rumen liquid. The pH of ryegrass and meadow fescue silages were on average 4.4 and 4.5, respectively. In both trials, inoculation increased silage pH and mechanical treatment reduced silage pH. The FAEI alone or along with mechanical treatment had no effect on NDFD of silages.
Keywords: ryegrass, meadow fescue, ensiling, *in vitro* incubation, feruloyl esterase, fibre digestibility.

Summary

Nowadays the world population is continuously growing, resulting in a great human-animal food/feed competition. This drives dairy cattle production to replace grain feeds by forages, with less/no impairment of the animal performance. In temperate regions, forages are mainly conserved as silage, which is produced from crops containing high moisture content, preserved and stored through airtight mechanism. Silage contains high fibre contents. Fibre is needed for a normal function of digestive tract of the animal and is the main energy source in dairy diet, although it has relatively low digestibility. Therefore, intensive researches are conducted worldwide to improve fibre digestibility. There are some bacteria that have ability to produce a specific type of enzyme that can potentially improve fibre digestibility. The objective of this experiment was to investigate the effect of inoculation with this type of bacteria along with mechanical treatment before ensiling on fibre digestibility of grass silage. Ryegrass and meadow fescue samples were collected, chopped and ensiled in laboratory scale silos. The treatments were control, inoculation, mechanical treatments and combination of inoculation and mechanical treatments. Unfortunately, treatments had no effect on fibre digestibility of grass silages.

Keywords: ryegrass, meadow fescue, ensiling, *in vitro* incubation, feruloyl esterase, fibre digestibility.

Table of contents

List of tables

List of figures

Abbreviations

ADF	Acid detergent fibre
ADFD	Acid detergent fibre digestibility
CFU	Colony forming units
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
FAE	Feruloyl esterase
FAEI	Feruloyl esterase producing inoculant
LAB	Lactic acid bacteria
ND	Neutral detergent
NDF	Neutral detergent fibre
NDFD	Neutral detergent fibre digestibility
OM	Organic matter
peNDF	Physically effective neutral detergent fibre
VOS	*In vitro* organic matter digestibility

1 Introduction

The issue of food security receives considerable attention for the coming decades as the world population will rise and demand for animal origin foods will become higher. Hence, an effort to fulfil this demand can create a huge burden on the food system of the globe (Smith *et al.* 2013). Increasing productivity of livestock will have great contribution in feeding the ever-growing population of the world (Estrada *et al.* 2011). Likewise feeding to animals is the major input cost in almost all animal farming systems (Archer *et al.* 1999) and in the future years costs of cereal grains are likely to stay elevated (Guyomard *et al.* 2013). For this reason, dairy cattle production is on urge to substitute grain feeds with forages, with less/no impairment of the animal performance. Lignocellulosic biomass is the major component in dairy cattle feed and is an important resource for the production of biofuels and biochemicals. Since lignocellulosic biomass in most cases are relatively low in digestibility, intensive researches are conducted in various corners of the world to boost its digestibility. According to Van Soest (1994), digestibility of fibre is less than 60%. In ecosystems like the rumen or biogas reactors, hemicellulose and cellulose can be degraded but lignin cannot be degraded. In cell walls of grasses and cereals, hemicellulose and lignin connect together mainly via ferulic acids. The lignin-hemicellulose matrix covers cellulose and this in turn makes the fibre fraction resistant to utilization(Rubin 2008; Pu *et al.* 2013). Feruloyl esterases (FAE) (EC 3.1.1.73) can break connections between lignin and hemicellulose, by which, bioavailability of cellulose and hemicellulose is enhanced (Addah *et al.* 2012). However, results from this approach have been inconsistent. For example, experiment by Nsereko *et al.* (2008) and Kang *et al.* (2009) improved NDFD of silage inoculated with a FAE producing *Lactobacillus buchneri* strain while an experiment by Lynch *et al.* (2014, 2015) failed to improve NDFD of silage treated by the same inoculant. One possible explanation could be that bonds between hemicellulose and lignin are not easily accessible by the FAE, due to complexity of cell wall structure. Thus, physical treatments of forage before ensiling with FAE producing LAB could enhance accessibility of these bonds.

In temperate regions, forages of high-water contents are mainly stored as silage. The aim of this work was to investigate the effect of a FAE producing inoculant along with physical treatment prior to ensiling on digestibility of silage fibres.

2 Literature review

2.1 Silage

Silage is a fermented product from anaerobic storage of high moisture content crops. The most common crops conserved as silage are cereal crops, legumes and grasses. For a successful ensiling, the first objective is to achieve anaerobic conditions. The most effective method is to keep the crop in a completely airtight container throughout the ensilage and prevention of air re-entry and circulation to the silo during storage. When herbage has long-term contact with oxygen during ensiling, both molds and yeasts grow and consequently, the material decays and becomes useless, toxic and inacceptable by the animal (McDonald *al.* 1991). The second objective is to inhibit growth of harmful microorganisms such as enterobacteria and clostridia. The effective means to hinder the undesirable microorganisms is to promote growth of lactic acid bacteria (LAB) or to apply chemical additives. Silage making process has four stages: (1) aerobic respiration immediately after filling, (2) fermentation stage, (3) storage phase and (4) feed-out phase (McDonald *et al.* 2010).

2.2 Factors affecting silage fermentation and aerobic stability

To maximize silage nutritional quality and reduce DM loss, it is advisable to know the influential factors in order to apply better management practices. Silage fermentation quality and aerobic stability are influenced by several factors including crop type, crop water contents, silo loading rate and crop compaction level and sealing (Weinberg & Muck 1996; Johnson *et al.* 2003; Borreani *et al.* 2007).

2.2.1 Wilting

Nowadays, most forages are not conserved immediately after harvesting rather they are required for field wilting in order to decrease water content to improve their ensiling potential and prevent effluent losses (Borreani et al. 2018). Comparing wilted grass with immediately ensiled, DM content of the wilted silage was higher ($p < 0.05$) which limited fermentation of silage (less acetic and lactic acids concentration) and enhanced fermentation quality by lowering ammonia concentration (Zhao-hai et al. 2012). In the UK during the last 30 years, silage mean DM content

has increased by 8% (from 22% to 30%) (Finch et al. 2014). Wilting of different lengths showed highly significant effects on butyric acid, acetic acid, pH, and ammonia-nitrogen values ($p < 0.01$) while, on LAB, lactic acid content, mould and yeast counts, WSC, NDF concentration had significant effect ($p<0.05$) but had no significant influence on CP, ADF, ether extract and counts of aerobic bacteria ($p > 0.05$). This implies even though wilting has promising effect on silage fermentation quality, the length of wilting would have paramount importance (Liu *et al.* 2011).

Leaving the harvested forage for extended time in field could result in aerobic fermentation, which produces heat and decrease the nutritional quality of silage, cutting is accompanied with various types of conditioners to speed the drying process. According to Borreani et al.(1999), cutting along with diverse conditioners for Italian rye grass has visible effects in increasing wilting rates with reduced field DM losses, which was below 2%. While the field DM losses of alfalfa was 1.1 to 3.3% with conventional conditioner (rubber machine) and 3.6 to 10.2% with the severe mower-conditioner (steel fail machine) and also the steel fail machine conditioning treatment resulted in more than 20% CP losses. Therefore, they concluded that a mild conditioning without tedding is more suitable to wilt alfalfa to prevent excessive leaves field losses. On the other hand, the harshest conditioning accompanied with tedding is more proper wilting treatment for grass, as it can significantly shorten the wilting duration with no significant effect on field losses.

2.2.2 Crop type

Crops having fermentable carbohydrates are easy to ensile and there is no need for additive treatments. Some of the crops that fulfil this parameter are whole-crop cereals with high starch concentration and tetraploid or Italian ryegrass that have high sugar contents. Crops with low sugar contents such as legumes and short leafy grasses at their peak growth stage demands extra treatment for successful fermentation, such as wilting, effective additives application or both (Finch *et al.* 2014). Study by Yahaya *et al.* (2004) on silage fermentation quality of temperate Italian ryegrass and tropical elephant grass showed the Italian ryegrass silage had a higher fermentation quality than that of elephant grass. One reason could be the Italian ryegrass has more sugars. Crops with low contents of fermentable carbohydrates have a slow pH drop during ensiling process, which allows the occurrence of secondary fermentation. In the secondary fermentation, saccharolytic clostridia, ferments acetic acid, lactic acid and residual sugars. This results in an increased concentration of butyric acid and pH rise. In addition, amino acids are also fermented

to NH_3 and amine, which further reduces the nutritional quality of silage (Pahlow *et al.* 2003).

2.2.3 Exposure to Oxygen

When oxygen enters into the silo, aerobic microbes, primarily yeasts, start consuming acids and sugars, consequently pH and temperature of the silage rise (Pahlow *et al.* 2003). As pH rises, aerobic bacteria and bacilli grow, temperature increases further; then moulds start growing which further deteriorate silage quality. If there is aerobic deterioration, the efforts made to produce a high quality silage is nullified (Borreani *et al.* 2018).

Slow silo filling and late sealing adversely affects the silage quality. Brüning *et al.* (2018) stated delayed sealing for about four days resulted in 11% DM losses, an increase in yeast population, declining of WSC contents by 65% of the forage before ensiling. It also results in ethyl esters formation during the fermentation time, that can reduce the palatability of the forage. Late sealed silos (with no additive) had lower lactic acid contents compared with prompt sealed silos (with no additive) (Weiss *et al.* 2016). This delayed sealing may promote extended aerobic respiration by the crop enzymes or numerous aerobic epiphytic microbes that compete for readily fermentable carbohydrate with the LAB (McDonald *et al.* 1991; Pahlow *et al.* 2003). When the packing is delayed, it also increases the activity of heterofermentative LAB and enterobacteria which leads to high acetic acid concentration (Weiss *et al.* 2016).

An experiment was conducted by Borreani *et al.* (2007) to compare the efficiency of conventional polyethylene with a recently developed oxygen barrier in silage quality. The study revealed the new oxygen barrier improves silage aerobic stability and reduces DM loss during fermentation, storage and fed-out phases. Comparing to the surface losses during the storage phase, contact to oxygen in the feed-out phase is stronger, once the silo is unsealed air penetrates up to four m deep into the silage particularly the surface part of the silo (Vissers *et al.* 2007). Nowadays it is well understood that feed-out phase is equivalently as important as the fermentation and stable phases from the point of maintaining good silage quality and nutrient preservation perspective (Borreani & Tabacco 2010; Driehuis 2013).

2.2.4 Weather

High temperature in the course of ensiling and rain at harvesting negatively affect fermentation and aerobic stability of the silage (Garcia *et al.* 1989). Proteolysis and effluent production can occur when it rains during crop collection (McDonald,P. *et al.* 1991; Fransen & Strubi 1998). Elevated temperature at ensiling decreases concentration of lactic acid and aerobic stability, as well increases DM loss and pH value (Ashbell *et al.* 2002). Both the fermentation speed and microbial species that lead fermentation are affected by temperature during ensiling. Ensiling at 40°C increased pH, concentration of residual WSC and ammonia-nitrogen; and hence resulted in a lower lactic: acetic acid ratio, which implies poor fermentation, high secondary fermentation and proteolysis. Overall, this experiment revealed that corn silage fermentation is negatively affected by high ensiling temperature (Kim & Adesogan 2006). However study by Weiss *et al.* (2016) found profound effect of high temperatures (35 vs. 20°C) on the improvement of aerobic stability.

A trial was carried out to evaluate minimum temperature needed for successful fermentation of whole-crop maize silage (Pauly 2010). The silages were made at 18, 12, and 6°C in the first year and 21, 14, 7 and 3°C in the second year. Taking into account the information shortage about 2-3°C treatment, a temperature of 6°C seemed to be the minimum temperature for whole-crop maize ensilage. At 6°C the silage pH was four in 60 d of fermentation. However, these silages had higher ethanol and lower acetate and lactate than silages kept at elevated temperatures (Pauly 2010). In addition, grasses in sunny weather are likely to have high sugar levels. Maximum grass sugar or WSC level has been recorded in grasses harvested in the afternoon of a sunny day (Finch *et al.* 2014). This reflects weather can directly affects the constituents of the live plant and as a means affects positively or negatively the fermentation quality of the respective silage.

2.2.5 Silage additives

Microbial inoculates

Previously it was believed that natural LAB count of forages is sufficient to assure well-fermented silage. But later it becomes clear that many crops have insufficient levels of LAB and others have even detrimental strains for proper silage making

process. Effectiveness of microbial inoculants on silage fermentation is dependent on the levels of WSC and inoculation rate, recommended to be higher than log 5 cfu/g fresh crop (McDonald *et al.* 2010).

Inoculation of fresh forage with homofermentative and heterofermentative LAB increases fermentation of WSC; as a result, produces enough amount of lactic acid to drop the pH and inhibit the effect of detrimental epiphytic microorganisms and maintain the nutritional quality of the silage (Ogunade *et al.* 2016; Silva *et al.* 2016). On the other hand, homofermentative or facultative heterofermentative LAB inoculants are poor in maintaining aerobic stability of the silage during open phase, as these inoculants produce lower amounts of acetic acid, an inhibitor of yeasts and molds growth, while, produce more lactate which can be the substrate for yeast growth (Weinberg *et al.* 1993). Nevertheless, inoculation with facultative heterofermentative or homofermentative LAB has encouraging effect on animal performance and silage fermentation although their degree of effectiveness relies on the species of inoculant, forage type; and related management activities during ensiling (Weinberg & Muck 1996).

In one study, crops inoculated with facultative heterofermentative LAB had a low pH both in tropical and temperate grasses and legumes. All the inoculated crops (excluding alfalfa) had lower acetic acid concentrations than the controls (Oliveira *et al.* 2017). In addition to improving silage fermentation, these bacteria also improve feed efficiency, daily weight gain and milk production (Weinberg & Muck 1996).

At mid1990th, aerobic stability improving inoculant (*L. buchneri*) reached to the market. This inoculant is an obligate heterofermentative LAB species that produces acetic acid and 1,2-propanediol via anaerobic fermentation of lactic acid thus, improves successfully aerobic stability of various silage types (Oude Elferink et al. 2001). The efficiency of the *L. buchneri* depends on the strain and application rate of the inoculant (Taylor & Kung 2002; Kleinschmit et al. 2005). According to the meta-analysis by Kleinschmit & Kung (2006) the aerobic stability of corn silage inoculated with >100,000 cfu of *L. buchneri*/g of fresh forage was 503 h, while it was 35 h for corn silage inoculated with ≤ 100,000 cfu of *L. buchneri*/g of fresh crop, but for the control treatment, it was only 25 h. There is a concern by some studies for DMI reduction because of the high production of acetic acid. However, (Kristensen et al. 2010) reported no negative effects on milk production, reproduction, health and intake comparing silage treated with *L. buchneri* 40788 to control silage.

As *L. buchneri* have saccharolytic and fibrolytic enzymes, silages inoculated with *L. buchneri* had reduced contents of NDF and ADF compared to silages without inoculation. However, the fibre concentration of the silages treated with the highest application dose of *L. bucheri* did not differ from the untreated silage (Kung & Ranjit 2001). Studies showed that lactic acid conversion to acetic acid anaerobically by *L. buchneri* demands 1-2 months. 1,2-propanediol which is an indicator of lactic acid conversion to acetic acid was not manifested in five days of fermentation, but it was occurred after 45 days of fermentation in silage inoculated with *L. bucheri* 40788 (Kleinschmit & Kung 2006).

Generally, a mixture of facultative heterofermentative and homofermentative LAB as inoculant can rapidly decrease the pH in the early weeks of fermentation and then during the stable and open phase, *L. buchneri* converts lactic acid to acetic acid that increases stability of the silage (Muck *et al.* 2018).

Chemical additives

chemical additives include acids and their salts. Examples of acids are propionic, benzoic, acetic, formic and sorbic acids. Perennial ryegrass silage treated with 0.1% sorbic acid had improved fermentation with reduced concentration of propionic, acetic and butyric acids, ammonia-nitrogen and ethanol (Shao *et al.* 2007). Improvements in silage fermentation quality by addition of chemical additives resulted in reduced DM losses. Such as, formic acid mixed with ammonium formate applied to wilted or direct cut grass silages at rate of 3 to 6 L/t caused reduction in the pH via direct acidification, a limited WSC fermentation, and diminished proteolysis and acetic acid formation (Saarisalo *et al.* 2006; Conaghan *et al.* 2012; Seppälä *et al.* 2016). Similarly, first cut timothy-meadow fescue grass silage ensiled with formic acid at 5 L/t had a restricted silage fermentation, lower ammonia-nitrogen and higher WSC content ($p<0.001$) compared to silages with no additives or treated with stabilized aqueous solution of hydrogen peroxide-sodium benzoate (Heikkila *et al.* 2012).

The impact of chemical additives on the silage quality could depend on the crop type. For instance, effect of formic acid-based treatment is crop specific. Addition of formic and propionic acids mixture to maize silage promoted high ethanol and ethyl ester formations but a mixture of formic acid and nitrite/hexamine declined ethanol production and ethyl esters concentration in lupin-wheat silage (König *et al.* 2017).

Application of mixture of several salts like potassium sorbate, sodium nitrite and sodium benzoate effectively enhanced quality of silage fermentation for forages having both low and high DM contents (Knicky & Spörndly 2011). Forages with less than 30% DM content treated with these salt mixtures had a reduced clostridial growth and consequently formation of butyric acid and ammonia was decreased. In crops with moisture content less than 65% treated with similar additive mixture, yeast activity was effectively eliminated in the silages upon silo opening phase. In another work, treatment of crops with salt-based additives at ensiling improved aerobic stability and reduced DM losses of silage (Knicky & Spörndly 2011). Addition of formic/propionic acid limited proteolysis and fermentation which in turn improved feed conversion efficiency, DMI and daily live weight gain of animals (Winters *et al.* 2001). Grass silage treated with 3.3 L/t formic acid fed to 400-kg Charolais X Friesian steers increased DMI from 7.4 to 8.4 kg/d and live weight gain per day from 0.67 to 0.94 kg, resulting in an improved feed conversion efficiency by 26% (Winters *et al.* 2001). The animal performance improvement was due to enhanced amino acid balance by attaining rapid pH drop during ensiling, hindering enzyme activity and inhibition of proteolytic bacteria (Winters *et al.* 2001). Alfalfa silage treated with ammonium tetraformate (7 L/t), compared to control treatment showed reduced protein proteolysis, evidenced from a reduced concentration of ammonia-nitrogen, soluble NPN and free AA-N (Broderick *et al.* 2007). Cows fed the treated silage increased DMI by one kg per day resulted in increased true protein contents of milk, meaning a better utilization of ingested N (Broderick *et al.* 2007).

Enzymatic additives

A number of enzymes have been added to forage at ensiling to enhance quality of fermentation and maintain silage nutritive value. Fibre digesting enzymes (cellulase and hemicellulase) could partly degrade the fibre portions of the plant (cellulose and hemicellulose), supplying WSC for fermentation of LAB., Similarly, addition of enzymes such as pectinase, b-glucanase, glucanase to maize and alfalfa forages at ensiling increased lactic acid and reduced ammonia, butyric acid and pH and thereby, improved silage fermentation quality (Dehghani *et al.* 2012). Even though fibre digesting enzymes can improve silage digestibility, extensive release of readily fermented carbohydrates could also increase risks of aerobic deterioration as residual WSC can be used by molds and yeasts (Kung and Muck, unpublished data). Application of cellulase and xylanase mixture along with a FAE producing inoculant to corn forage at ensiling resulted in a lower pH value and greater WSC of silage than the enzymes mixture treatment alone. Also, the silage

treated with enzyme mixture treatments only displayed elevated yeast counts and lowered DM recovery (Lynch *et al.* 2015).

2.3 Fibre fraction of grasses

Fibre is defined as the organic part of feeds with low digestibility, while non-fibre is portion of feeds that is easily and almost entirely digested by most animals (Mertens 2002). Plant fibre, found in the cell walls, is divided in to two parts: soluble or insoluble in the neutral detergent (ND). The insoluble part, known as NDF, is mainly the crosslinked matrix that forms the rumen mat, promoting normal rumen function, and comprises mainly lignin, cellulose and hemicellulose (Van Soest *et al.* 1991).

2.3.1 Fibre requirements

Fibre plays a great role in maintaining healthy rumen and keeping standard milk fat in dairy cattle. Enough rumination and cellulose degradations are reflections of normal rumen performance in dairy cow which help to buffer the rumen pH and maintain cellulase producing microorganisms. This results in a greater acetate to propionate proportion in the rumen needed for normal lipid metabolism (Van Soest *et al.* 1991). For animals fed easily digestible feed, fibre inclusion induces chewing and as a result more saliva with bicarbonate will flow to the rumen and dilute the acids released during ruminal fermentation. Hence, risk of subacute rumen acidosis is decreased and feed digestibility and utilization is improved (Allen 1997).

In addition to NDF amount and particle size, net fermentation rate, cation exchange and buffering capacity, amount of nonfibrous carbohydrates, ratio of starch to non-starch polysaccharides and protein supply have key effects on the rumen environment and microbes efficiency (Van Soest *et al.* 1991). Fibre is the main energy source in ruminants' diet. About 70% of energy requirement of ruminants is supplied from volatile fatty acids produced in the rumen (Bergman 1990). As major parts of volatile fatty acids are produced from the ruminal degradation of fibre, improving fibre digestibility is important (Bergman 1990).

2.3.2 Fibre content evaluation

Fibre analysis was conducted for the first time in 1860 by the method called proximate analysis at Weende laboratory in Germany. Fibre was measured as crude fibre where acid and alkaline were applied to filter the fibre fraction. Hemicellulose, as a part of fibre fraction, is degradable by acid, thus, crude fibre underestimate the amount of fibre. To fill this gap original NDF analysis procedure was introduced by Van Soest & Wine (1967) as described in (Van Soest *et al.* 1991). However, the original NDF method was unable to remove the starch from starchy feeds, hence this method was modified via inclusion of amylase (Robertson &Van Soest, 1981) see in (Chai & Udén 1998).

In the standard procedure by Van Soest *et al.* (1991), samples are boiled with ND solution. An alternative method was developed by Chai & Udén (1998) in which samples are incubated overnight with ND solution. The authors also suggested 25% strength ND solution can be used for feeds with low contents of protein but for high protein content feedstuff, the standard procedure of (Van Soest 1991) is more appropriate. As some samples can be contaminated with soil and soil is ended up in the NDF fraction, NDF can be corrected for the ash content to avoid this issue.

2.3.3 Constituents of fibre fraction

Grasses are the main source of dairy cattle feed. In order to utilize grasses efficiently, it is important to have good understanding about the physical, chemical and biological characteristics of grass cell wall. There are two types of cell walls called as primary and secondary cell walls. The growing cells of plants are surrounded by primary cell walls, which comprise mainly cellulose, hemicellulose and pectins. When the cells growth is ceased, they develop a secondary cell wall (structural carbohydrates plus lignin) to obtain additional strength (Scheller & Ulvskov 2010). The secondary plant cell wall comprises mainly lignin (10-30 %), cellulose (30-50 %) and hemicellulose (15-35 %).

Cellulose is a common constituent to all plants, but its level varies among plants. It is a single polymer which is available in large quantity in the plant kingdom. Cellulose is formed from repeating single glucose units linked by ß -1,4 glycoside linkage which in turn, reduces solubility of cellulose. Cellulose chains (molecules) formed

together by inter- and intra-molecular hydrogen bonding to form condensed aggregates called microfibrils (Gardner & Blackwell 1974; McDonald *et al.* 2002).

Hemicellulose is one portion of the plant cell wall solubilized in alkali. It is a heterogeneous carbohydrate composed largely of D-xylose, D-Mannose, D-galactose, D-glucose and arabinose units, tied together in numerous combinations and through diverse glycoside linkages (McDonald *et al.* 2002). Some researchers recommend the term hemicelluloses instead of hemicellulose because this group of polysaccharides is still not well understood in terms of structures and biosynthesis. Nevertheless, it is generally agreed to use the term hemicellulose/hemicelluloses for a group of structural carbohydrates that is neither cellulose nor pectines and having β-(1→4)-connected backbones of Mannose, xylose or glucose (Scheller and Ulvskov, 2010). Ferulate esters are important bonds that cross-linked covalently hemicellulose to lignin. These cross-linking ferulate esters reduce ruminal degradation of hemicellulose and cellulose (Scheller & Ulvskov 2010).

Another important part of the plant cell wall is lignin, which is not a carbohydrate. Lignin does not represent a single well-defined compound. Next to cellulose, lignin is the largely abundant natural polymer in the world. Grass lignin polymers are made up of three major unit types, guaiacyl, hydroxyphenyl, and syringyl units linked via biphenyl ether bonds (4-O-5 and 5-O-4), aryl ether bonds (ß-O-4 and α-O-4 linkages), and/or resistant carbon- carbon bonds (ß-5, ß-ß and 5-5). Ferulic acid, p-coumaric acid and p-hydroxycinnamates are also ester or ether linked to lignin. Lignin receives a great emphasis in animal nutrition since it is not digestible by digestive enzymes of animals. Lignin seals the open spaces within hemicellulose and cellulose and this in turn hinders digestibility of cellulose and hemicellulose (McDonald *et al.* 2002; Ralph *et al.* 2004).

2.3.4 Factors affecting fibre digestibility

Plant cell wall elements are analysed not only to recognize their structure but also to know and evaluate their nutritive value. When NDF digestibility of forage is high, DM intake and milk production of cows is also increased. Thus, forage NDF digestibility must be quantified to evaluate quality of forage (Oba & Allen 1999).

Plant maturity

Forage digestibility reduces as the maturity of the plant increases; this is because of the increased proportion of lignin in the plant cell wall. Therefore, it is advised to consider maturity stage of forage at harvesting to balance the grass yield and digestibility (Van Soest 1994). According to an experiment by Bosch *et al.* (1992) grass silages with higher maturity had higher share of cellulose, hemicellulose, lignin and lower crude protein content compared to silages from grass harvested at younger age. Both cellulose and hemicellulose digestibility were decreased as the cell wall percentage increased. The digestibility variations indicate that when the maturity stage of forage increases the amount and distribution of lignin in the cell wall of plant also increase. The lignin is cross-linked to cellulose and hemicellulose which protects them from degradation by the rumen microbes. As a result, digestibility of structural carbohydrates in the cell wall is reduced with plant maturity (Engels 1987).

Cutting number has also influence in NDFD of the forages. Forages from a higher cutting number have a lower digestibility compared to forages with a lower cutting number. An experiment by Dohme *et al.* (2007) revealed cutting number of English ryegrass, Italian ryegrass, red clover and lucerne had an influence on NDF degradability where NDF degradability of second cut forage was lower than the first cut forage.

Fibre source

Degradability of different grasses varies for many reasons. Temperate grasses are more degradable than tropical grasses. This is because temperate grasses contain more mesophyll, phloem cells and less lignin content as well as less parenchyma and epidermal bundle sheath cells (Akin,1986) as described in (Buxton & Redfearn 1997).

Digestibility and utilization of fibre from different forges are different. Degradability of lignified cell wall in grasses is extensively higher than that of in legumes with similar lignin thickness. The reason for differences in degradation can be revealed by structural analysis of cell walls of legumes and grasses (Jung & Engels 2001). Another study argued that even though legume fibres are more lignified and are less digestible than that of grasses, legume forages are better degraded than grass forages because legumes have less fibre (Buxton & Redfearn 1997). However (Beever *et al.*

1985) reported that ruminal digestibility of OM and cellulose of perennial ryegrass was higher than fresh white clover in cattle fed these two diets.

Lignin composition, lignin content and cell wall crosslinking are the main factors that have great impact on the digestibility of forages (Jung 2012). Even within a single plant species genotypic variation exists in lignification thus digestibility differs accordingly (Moore & Jung 2001a).

Animal genetic make-up

Cows of different breeds have diverse efficiency in grass digestion (Berry *et al.* 2007). The NDF and ADF digestion was lower in Holstein lactating cows compared to Jersey and Jersey x Holstein Friesian cows. Jersey and Jersey x Holstein-Friesian cows had larger rumen size than Holstein dairy cows, which could be the reason for the effect observed (Beecher *et al.* 2014). Passage rate is lower when the rumen size is larger. This bigger size of the rumen can give more time to rumen microbes to adhere and degrade the feed (Beecher *et al.* 2014). Similarly, beef steers with better digestion capacity showed 10% feed conversion efficiency difference (Richardson & Herd 2004).

2.3.5 Methods to improve fibre degradability in the rumen

Physical feed treatment

It is hard for rumen microbes to reach the interior layers of the lignified plant cells unless cells are physically raptured (Jung *et al.* 2012). As a result, one-third of the cells of the grass pass the rumen without digestion (Jung *et al.* 2012). An experiment by Weisbjerg *et al.* (2018) was conducted to find out impact of pre-ensiling physical treatment on grass-clover silage fibre digestibility. The plant was shredded by a shredder, without being chopped and then was ensiled. The ruminal digestibility of silage fibre was improved. This could be due to the physical damage of the cell wall of the silage that gave a chance to rumen microorganisms to directly adhere to the fibre (Hong *et al.* 1988). However, in a study conducted with ryegrass silage, maceration prior to ensiling decreased fibre digestibility. This could be because of a higher leaf losses during maceration (Broderick *et al.* 2007).

According to Bal *et al.* (2000) feed processing of corn silage can affect total tract digestibility of fibre. The authors observed reduced NDF and ADF digestibility with finely processed silages (0.95 cm length).

Microbial treatment

Previous studies showed that feruloyl esterases can unlock the association between hemicellulose and lignin of plant fibres and this can in turn increase ruminal degradation of fibres (Nsereko *et al.* 2008). Several species of *Lactobacillus* genus are shown to produce the feruloyl esterase (Donaghy *et al.* 1998). An experiment was conducted by Nsereko *et al.* (2008) to study the effect of FAE producing inoculants on the NDF digestibility of perennial ryegrass and whole plant corn silages. The authors tested several FAE producing strains and found increased in *in situ* NDFD of silages. In other study, NDFD of vigoro61R36 corn cultivar inoculated with FAEI was improved by 11% compared to the untreated silage (Kang et al. 2009). A further work by Jin *et al.* (2015) with barley silage and using *L. buchneri* mixture inoculants as FAEI showed similar results on NDFD. On the other hand, there are some works in which this approach did not improve NDFD. In the work of (Lynch *et al.* 2014, 2015) in which alfalfa and corn, respectively, were ensiled with FAE producing strain of *L. buchneri*, no effect was found on fibre degradability. Silage inoculants can also act as probiotics, meaning the silage can deliver specific type of microbes to the rumen (Weinberg *et al.* 2004).

Enzymatic treatment

Plant cell wall digesting enzymes (hemicellulases and cellulases) are the most known enzymatic additives used for silage production. They are often added to silage to provide substrates for silage fermentation. In this matter, application of cell wall degrading enzymes is more relevant for tropical crops as these crops have generally low sugar contents (Nadeau *et al.* 2000; Khota *et al.* 2016). There is also evidence that fractional damage of the plant cell wall by these enzymes could en-

hance ruminal degradability of silage (Kung, 2014). To increase their effects, cellulase and hemicellulase are usually applied together. In a study on (Guo *et al.* 2014), *in vitro* fibre digestibility and fermentation quality of mixture of whole-crop corn and barley straw were improved when fibrolytic enzymes comprising xylanases and cellulases were applied at ensiling. Addition of fibrolytic enzymes together with bacterial inoculants has also shown promising results in increasing *in sacco* NDF solubility, silage fermentation quality and enhancing feed efficacy of beef cattle (Zahiroddini *et al.* 2004).

On the other hand, there are some instances in which application of fibrolytic enzymes reduces fibre digestibility of silages. In an experiment by Nadeau *et al.* (2000), NDFD of orchard grass and alfalfa silages treated with cellulase was reduced by 18%. Similarly, (Jaakkola *et al.* 1990) found a reduced NDFD of silage treated with cellulase at ensiling. One possible explanation is that under certain conditions, easily digestible parts of the NDF are degraded during ensiling, leaving less degradable fibre for rumen microbes (Nadeau *et al.* 1996).

Enzymes can also be added directly to feed before ingestion, which can enhance removal of the structural barriers that hinder the efficiency and discharge of soluble carbohydrates from the cell wall of the plant in the rumen. As a result, the rumen microorganisms will have more chance to directly contact with the soluble carbohydrates and digest them (Beauchemin *et al.* 2004).

Plant lignin reduction through breeding/mutation

Lignin concentration has negative correlation with fibre digestibility of forages hence breeding and mutation could be used to reduce lignin contents of plants. A typical example is brown-midrib maize, a mutated variety, with altered composition and reduced concentration of lignin comparing to the normal genotypes of maize. As a result, fibre digestibility of this maize variety was improved (Cherney *et al.* 1991).

According to Jung and Deetz (1993) as described in (Moore & Jung 2001b) the key reason for lignin to hinder grass cell wall digestibility is the ferulate cross-linking of lignin to structural carbohydrates. Therefore, to enhance utilization of structural carbohydrates, genetic engineering and breeding can be implemented to produce grasses with low lignin-structural carbohydrates cross-linking (less ferulate acids). It was previously shown that direct selection of grasses for lower ferulate crosslinking of -lignin to structural carbohydrate resulted in improvement of bromegrass cell wall degradation (Grabber 2005).

3 Material and methods

3.1 Grass preparation

One sample of 2nd-cut ryegrass (Lolium perenne) and one sample of 2nd-cut meadow fescue (Festuca pratensis) were collected in Uppsala, Sweden during Autumn 2018. Heads of ryegrass sample were fully developed whereas meadow fescue had no heads. Samples were chopped by a stationary chopper to an approximate length of 5 cm and stored at -20°C until the experiment was started in the spring 2019.

3.2 Inoculant preparation and analysis

The *Lactobacillus buchneri* LN4017 (ATCC no. PTA-6138) was cultured in 9 mL MRS broth (Merck KGaA, Darmstadt, Germany) for 48 h at 37°C after which, the bacterial culture was centrifuged at 4000 × g for 5 min. The bacterial pellet was suspended in 1 mL suspensions of Ringer solution (Merck KGaA, Darmstadt, Germany) before storage at -80°C.

Viability and counts of the inoculant were checked prior to applying to the grass samples. The MRS agar (Merck KGaA, Darmstadt, Germany) plates were prepared according to manufacturer's instruction. One of the frozen bacterial stocks was thawed at room temperature and serial dilutions was prepared, with Ringer solution as diluent. An amount of 100 µL was spread on MRS agar plates and plates were incubated anaerobically at 37°C for 48 h.

To enumerate epiphytic LAB of forages, an amount of 20 g forage, in duplicate, was taken randomly from each crop before adding 180 mL Ringer solution and maceration for 2 min in a stomacher (Seward 3500, Seward Ltd, Worthing, UK). Serial dilution was prepared from the bacterial suspension and culturing was done on MRS agar plates as described above.

3.3 Ensilage

Grass samples were thawed and wilted in ambient temperature, hence the DM content of the samples reached ~35%. Six treatments, with three replications, were compared for their effect on NDFD of silages: untreated control, inoculation with *L buchneri*, mild mechanical treatment (Mild), harsh mechanical treatment (Harsh), inoculation plus Mild and, inoculation plus Harsh. The Mild treatment was hitting grass samples (200 g) with a 4.8-kg metal rod from a height of 55-cm for 100 times (Figure 1) and the Harsh treatment was mincing grass samples (200 g) with a meat mincer using a 12.8 mm die (Figure 1). For the inoculation, 5 mL bacterial solution was sprayed over 100 g forage sample and sample was mixed well before ensiling in glass tubes (100 mL). For the control, forage was sprayed with 5 mL Ringer solution. Silos were sealed with water-lock and stored at room temperature for 48-49 d. After silo opening, 15 g distilled water was added to 15 g silage before storage in a fridge overnight. In the following morning, silage juice was extracted by hydraulic pressure and pH of the silage juice was measured with a pH-meter (Metrohm 654, metrohm AG, Herisau, Switzerland).

3.4 Chemical analysis

Grass and silage samples were dried (60°C, 18 h) and milled by a hammer mill (KAMAS Slagy 200, Malmö, Sweden) with a 1-mm screen. DM, ash, and OM (organic matter) were analyzed according to the procedure by (AOAC 1990). To determine the DM contents, the semi-dried and ground samples were dried at 103^{o}C for about 18 h. The ash content was estimated by incineration at 550^{o}C for about 3 h. OM was calculated by subtracting ash from DM.

For the silage samples, as there are losses of volatiles during drying, the estimated DM was corrected using 1.577+0.992 × (DM%) as described by (Mogodiniyai Kasmaei *et al.* 2015).

Nitrogen concentration was measured by the Kjeldahl technique (Kjeltec 1030, Tecator, Höganäs, Sweden) and was expressed as CP by multiplication with 6.25.

WSC was measured by enzymatic method as described by (Udén 2010)

Figure 1. The metal rod and container used for mild mechanical treatment and the meat mincer used for harsh mechanical treatment.

Figure 2. Mechanically treated fresh ryegrass. From left to right: control, Mild and Harsh treatment.

Figure 3. Mechanically treated fresh meadow fescue grass: From left to right: control, Mild and Harsh treatments

NDF of grass prior to ensiling was measured according to (Chai & Udén 1998) by incubation with ND solution overnight and treatment with amylase and sodium sulfite before ashing.

3.5 *In vitro* NDFD of silage

Prior to the *in vitro* incubation to estimate NDFD of silages, silage samples were extracted by water to remove major parts of water-soluble nutrients. An amount of 5-g ground silage was incubated with 50 mL distilled water at 85°Cfor 2 h before drying the extracted residue at 60°C for 18 h. The DM content of the residues was measured by weighting 1-g and drying at 103°C for 18 h.

3.5.1 NDF concentration of water extracted samples

A quantity of 0.5 g dried residue was weighed in P2 glass filter crucibles (40-100 µm) fitted with rubber stopper on the bottom and 50 mL ND buffer solution was added. Crucibles were covered with aluminum foil and a few holes were made in the aluminum foil. The crucibles were incubated at 85°C for 22 h and agitated following 2-h incubation. Upon completion of incubation, the rubber stopper was removed, and the crucibles were drained and rinsed with approximately 20 mL hot distilled water. Afterwards, the crucibles were placed on a suction manifold and washed with hot distilled water until there was no foam. This was followed by washing twice with acetone before drying at 103°C for 18 h.

3.5.2 *In vitro* incubation

Gas Endeavour system (Gas Endeavour, Bioprocess Control AB, Lund, Sweden), with 15 incubation chambers (Figure 4) was employed for the *in vitro* incubation. The system allows real-time measurements of gas produced. Two *in vitro* incubation batches were run, one for the water extracted ryegrass silage and one for the water extracted meadow fescue silage. P2 glass filter crucibles were used as the incubation vessel. From each treatment, two replicates were connected to the gas measurement unit and one replicate was sealed with a water lock. Three negative controls (no substrate) were also included in each run, with two being connected to the gas measurement unit and one being sealed with water lock**.**

Preparation of in vitro incubation

VOS buffer solution (2 L) was prepared one day prior to the *in vitro* incubation. The proportion of ingredients per 1 L buffer solution was 8.50 g $NaHCO_3$, 0.50 g $(NH_4)_2HPO_4$, 5.80 g K_2HPO_4, 1.00 g NaCl, 0.01 g $FeSO_4$•7 H_2O, 0.50 g $MgSO_4$•7 H_2O, and 0.10 g $CaCl_2$. The ingredients were dissolved in distilled water. The

$FeSO_4$•7 H_2O and $CaCl_2$ were dissolved separately before adding to the buffer. The $NaHCO_3$ was added approximately 40 min prior to the onset of incubation to keep the buffering capacity and pH level in a needed range. The VOS buffer solution was placed in a water bath (38ºC) and were gassed with CO_2 overnight. An amount of 0.5 g DM of water extracted samples was weighed into P2 glass filter crucibles. Rumen fluid sample was obtained from a rumen fistulated Swedish Red and White breed, fed at maintenance level, 2 h after morning feeding. The cow was maintained under ethics approval by Uppsala Ethics Committee (C 142/14). The rumen fluid was transferred in a pre-warmed Thermos to the laboratory within 30 min, after which, it was filtered (1-mm screen) and 40 mL was added to 1960 mL buffer. Thereafter, 50 mL of diluted rumen fluid was poured into glass filter crucibles and crucibles were sealed as described above. The incubation was performed at 38°C for 96 h. The negative controls were used to correct gas production data and residual NDF for contribution from buffered rumen liquid. After terminating the incubation, the crucibles were drained, and pH of the incubation medium was measured by a pH meter. Residual NDF in the crucibles was estimated by incubation with 50 mL of ND buffer as described in section 3.5.1 with only difference of agitation of crucibles also in the morning before terminating the incubation.

Figure 4. An image of the experimental setup. The gas measurement unit of Gas Endeavour (Bioprocess Control AB, Lund, Sweden) was used to record the gas production.

Calculation

The NDFD was calculated as the portion of incubated NDF disappeared during incubation. DM loss during ensiling was calculated as the portion of silage DM disappeared during ensiling.

3.6 Statistical analysis

General Linear Model procedure of Minitab (Minitab®18.1, Minitab, Ltd., Coventry, UK) was used to analyze the data. The statistical model included a factorial procedure with two factors (inoculation and mechanical treatment) and the interaction of the two factors. The significant level was declared at $p < 0.05$. The Tukey method was used for pairwise comparison.

,

4 Result and discussion

4.1 Chemical and microbial composition

The chemical composition of ryegrass and meadow fescue is presented in *Table 1*. The pre-ensiling chemical composition of ryegrass was similar to that of (Jatkauskas *et al.* 2013) while for meadow fescue it was comparable to that of (Gregorini *et al.* 2009). The DM, NDF and WSC contents of ryegrass were 360 (g/kg), 437 (g/kg DM) and 70 (g/kg DM), respectively, while the DM, NDF and WSC of meadow fescue were 350 (g/kg), 455(g/kg DM) and 59 (g/kg DM), respectively. .

Table 1. Chemical composition (g/kg DM unless otherwise stated) of ryegrass and meadow fescue grasses before ensiling.

Item	Crops	
	Ryegrass	Meadow fescue
DM(g/kg)	360	350
Ash	119	125
OM	881	875
CP	193	198
NDF	437	455
WSC	70	59

DM= dry matter, OM= organic matter, NDF= neutral detergent fibre, CP = crude protein

Chemical composition of ryegrass and meadow fescue silages is presented in *Table 2 and 3 respectively*. In some replicates, the estimated DM loss was negative, and, in this case, the DM loss was considered zero. DM loss in one of the replicates of inoculation plus Harsh treatment in the meadow fescue trial was 12% higher than the average value of the other two replicates and thus, it was excluded from the analysis. This could have been due to an error during estimating the DM content of this silage replicate, which was 4% lower than the average of the other two replicates. This in turn resulted in calculating a lower DM content remained after ensiling for this replicate compared to other two replicates.

For ryegrass silage, the DM contents of silages treated with the inoculant were lower than the silages treated without inoculation. The DM content of silages treated with Harsh was lower compared to control and Mild treatments. However, the interaction effect of the inoculation and mechanical treatments was not significant. The DM loss did not show any significant differences in all treatment categories. For meadow fescue silage, DM content and DM loss did not differ by any of the treatments.

In general, the treatments had no effect on DM losses during ensiling. However previous studies showed inoculation with *L. buchneri* resulted in higher DM losses of silage during fermentation probably due to higher formation of CO_2, when lactic acid is converted to acetic and 2-propanediol (Driehuis et al. 2001; Oude Elferink et al. 2001).

The *L. buchneri* was added to both forages at 10^6 CFU/g forage. The number of epiphytic LAB of meadow fescue and ryegrass were 10^6 and 10^7 CFU/g forage, respectively.

4.2 Silage fermentation characteristics

The ryegrass and meadow fescue silages pH are in *Table 2 and 3* respectively. For both ryegrass and meadow fescue the inoculated silages had higher pH than the uninoculated. In both silages, Harsh treatment resulted in the lowest silage pH. On the other hand, pH of ryegrass silages produced with inoculation plus Harsh treatment was lower than silages produced with inoculation alone or inoculation-plus-Mild treatments. From these results it can be speculated that in latter two treatments, the inoculant dominated the fermentation. The *L. buchneri* is a heterofermentative LAB with ability to produce both lactic acid and acetic acid as well as converting lactic

acid to acetic acid, which resulted in a higher silage pH. This is in agreement with the trial conducted on ryegrass silage treated with *L.buchneri* alone which had higher pH (Driehuis *et al.* 2001; Oude Elferink *et al.* 2001). On the other hand, it seems that when the inoculation was accompanied by Harsh treatment, the epiphytic LAB dominated the fermentation. This could be because the Harsh treatment resulted in a faster release of silage substrate, by which, epiphytic LAB overtook the fermentation. In all treatments, silages had generally a low pH, which suggests that fermentation was successful.

There was no treatment effect on pH values after 96 h of *in vitro* incubation in both ryegrass and meadow fescue silages. The pH level during incubation is influenced by the quantity of organic acids produced and composition of the buffer. In the present study, the pH values of the incubation fluid were almost neutral (7.2 -7.3), indicating that rumen microbes functioned normally during 96 h incubation.

Table 2. Chemical composition (g/kg dry matter) of ryegrass silage together with estimated DM loss during ensiling (n=3). Values are least square means ±SEM.

Treatments		Variables				
		DM (g/kg)	Ash	OM	pH	DM loss
Inoculation (I)						
Inoculated		336^{b}	133^{a}	867^{b}	4.58^{a}	75
None		345^{a}	130^{b}	870^{a}	4.30^{b}	62
SEM		1.70	0.63	0.63	0.02	10.80
Mechanical treatment (M)						
None		344^{a}	129^{b}	871^{a}	4.56^{a}	49
Harsh		334^{b}	135^{a}	865^{b}	4.31^{c}	81
Mild		343^{a}	130^{b}	870^{a}	4.44^{b}	76
SEM		2.09	0.78	0.78	0.02	13.20
I ×M						
Inoculated	None	340	131	869	4.72^{a}	63
Inoculated	Harsh	331	137	863	4.39^{bc}	90
Inoculated	Mild	338	131	869	4.62^{a}	72
None	None	348	127	873	4.41^{b}	36
None	Harsh	337	133	867	4.23^{c}	72
None	Mild	348	128.0	872	4.26^{bc}	80
SEM		2.95	1.10	1.10	0.03	19
P value						
I		0.006	0.002	0.002	<0.001	0.430
M		0.009	<0.001	<0.001	<0.001	0.234
I×M		0.840	0.964	0.964	0.030	0.631

Mild=pounding with a metal rod, Harsh = mincing with a meat mincer, DM =dry matter, OM =organic matter. Values not sharing a superscript within a column are different ($p < 0.05$)

Table 3. Chemical composition (g/kg dry matter unless otherwise stated) of meadow fescue silage together with estimated DM loss during ensiling (n=3). Values are least square means ±SEM.

Treatments		Variables				
		DM (g/kg)	Ash	OM	pH	DM loss
Inoculation (I)						
Inoculated		344	132	868	4.59[a]	10[1]±4.74
None		346	131	869	4.39[b]	13±3.9
SEM		3.97	0.46	0.46	0.02	
Mechanical treatment(M)						
None		347	132	868	4.62[a]	16
Harsh		340	132	868	4.36[c]	9[1]±6.01
Mild		348	131	869	4.49[b]	10
SEM		4.86	0.57	0.57	0.02	5.38
I×M						
Inoculated	None	348	134a	866	4.72	17
Inoculated	Harsh	336	132ab	868	4.42	3[1] ± 9.31
Inoculated	Mild	348	130b	870	4.61	10
None	None	345	131ab	869	4.52	16
None	Harsh	345	132ab	868	4.30	14
None	Mild	349	132ab	868	4.37	11
SEM		6.88	0.80	0.80	0.03	7.60
P value						
I		0.698	0.403	0.403	<0.001	0.594
M		0.470	0.149	0.149	<0.001	0.623
I×M		0.655	0.032	0.032	0.271	0.738

Mild=pounding with a metal rod, Harsh = mincing with a meat mincer, DM =dry matter, OM =organic matter. Values not sharing a superscript within a column are different ($p < 0.05$). [1]Observations had missing values.

4.3 The effect of treatments on NDFD

The pre *in vitro* incubation NDF content of ryegrass silage (*Table 4*) was not affected by treatments whereas the NDF content in the post *in vitro* incubation of the inoculated silage were greater than the un-inoculated. The NDF concentration of meadow fescue silage samples pre and post *in vitro* incubation (*Table 5)* did not differ among treatments. In general, there was no treatments effect on NDFD of both ryegrass and meadow fescue silage samples. In the ryegrass trial, within inoculation-plus-Harsh treatment, one replicate was lost during post-incubation filtration. In the meadow fescue trial, for unknown reason, one of the replicates of inoculation-plus-Mild and one of the replicates of inoculation-plus-Harsh had considerably lower NDFD than the other replicates (10 and 6 times, respectively) and were thus removed from analysis.
In the current study, for unknown reason the mechanical treatment had no significant effect on fibre digestibility of the silage samples. However previous studies confirmed that fibre digestibility of grass-clover silage physically treated prior to ensiling was improved (Weisbjerg *et al.* 2018). This could be due to the physical damage of the cell wall of the silage that gave a chance to rumen microorganisms to directly adhere to the fibre (Hong *et al.* 1988).

In this study, the forages had high levels of epiphytic LAB. Grasses usually have low counts of epiphytic LAB (Mogodiniyai Kasmaei *et al.* 2015) but here, this was not the case. It is very likely that thawing of the frozen forages resulted in the release of forage substrates and this in turn triggered growth of epiphytic LAB during wilting. In the ryegrass trial, as it was mentioned above, it seems that the inoculant dominated the fermentation when it was applied alone, however, in this treatment, there was no effect on NDFD. This is in line with our starting hypothesis that the FAE produced does not reach target linkages between lignin and hemicellulose and therefore application of mechanical treatments is needed. However, when we applied the harsh mechanical treatments, it seems that the inoculant did not dominate fermentation in the ryegrass silage and thus, there was no effect on NDFD. In contrast to our trial, previous studies by Nsereko *et al.* (2008) and Addah *et al*. (2012) confirmed inoculation of grass or barley with FAEI at ensiling improved ruminal NDFD of silages through increasing the content of potentially degradable NDF portion in the silage.

It should be taken into account that the NDFD values obtained here may not reflect on the true NDFD values of these forages. The procedure we used is not a standard NDFD assay and therefore data generated are only to compare the effect of treatments within each forage type and not across forages.

Table 4. Neutral detergent fibre (NDF) pre and post *in vitro* incubation together with NDF digestibility (NDFD) of ryegrass silage (n=3). Values are least square means ±SEM and unit is g/kg dry matter.

Treatments		Variables		
		Pre-incubation NDF	Post-incubation NDF	NDFD
Inoculation (I)				
Inoculated		696	201[1]±5.87[a]	711[1]±9.14
None		690	180±5.43[b]	738±8.46
SEM		13		
Mechanical treatment(M)				
None		692	187	729
Harsh		703	199[1]±7.44	718[1]±11.60
Mild		685	187	726
SEM		15.90	6.65	10.40
I×M				
Inoculated	None	718	196	727
Inoculated	Harsh	704	211[1]±11.5	701[1]±18
Inoculated	Mild	666	197	704
None	None	666	179	730
None	Harsh	702	186	735
None	Mild	703	176	749
SEM		22.50	9.41	14.70
P value				
I		0.749	0.023	0.051
M		0.709	0.451	0.782
I×M		0.183	0.923	0.379

Mild=pounding with a metal rod, Harsh=mincing with mincer. Values not sharing a superscript within a column are different ($p < 0.05$). [1]Observations had missing values.

Table 5. Neutral detergent fibre (NDF) pre and post *in vitro* incubation together with NDF digestibility (NDFD) of meadow fescue silage(n=3). Values are least square means ±SEM and unit is g/kg dry matter.

Treatments		Variables		
		Pre-incubation NDF	Post-incubation NDF	NDFD
Inoculation(I)				
Inoculated		715	358[1]±19.60	496[1]±27.90
None		712	335±17.00	530±24.20
SEM		8.03		
Mechanical treatment(M)				
None		704	346±20.80	508±29.60
Harsh		719	338[1]	531[1]
Mild		719	356[1]	502[1]
SEM		9.84	23.20	33.10
I×M				
Inoculated	None	695	370	466
Inoculated	Harsh	715	335[1]±36.00	534[1]±51.20
Inoculated	Mild	736	370[1]±36.00	489[1] ±51.20
None	None	712	321	549
None	Harsh	723	341	528
None	Mild	703	341	515
SEM		13.90	29.40	41.80
P value				
I		0.791	0.383	0.379
M		0.461	0.869	0.812
I×M		0.204	0.684	0.609

Mild=pounding with a metal rod, Harsh=mincing with mincer, Values not sharing a superscript within a column are different ($p < 0.05$). [1]Observations had missing values.

4.4 Gas production from ryegrass and meadow fescue silages

Cumulative gas production data over the course of 96 h incubation of ryegrass and meadow fescue silages are presented in *Tables 6 and 7*, respectively. There was a large variation within replicates of each treatment and as a result, we could not detect the effect of treatments, if any, on gas production. The total gas volumes produced from the untreated control, inoculation-plus-Mild and inoculation-plus-Harsh treatments in the meadow fescue trial became negative after correction for contribution from the incubation medium. It can be speculated that substrates in these treatments had some kind of unknown inhibitory effect on microbial degradation. One likely reason for the large variation within each treatment could be the total volume of incubation medium (i.e. 50 mL) used was small and as a result, the gas produced was not adequate for the Gas Endeavor machine to accurately measure the gas.

.

Table 6. Gas volume (mL) (mean±SEM) from ryegrass silage (n=2) during 96 h *in vitro* incubation with buffered rumen liquid. Gas from buffered rumen liquid without substrate (n=2) was used as baseline.

Treatments		Hours				
		12h	24h	48h	72h	96h
Inoculation(I)						
Inoculated		1.65	4.35	10.15	16.45	16.98
None		0.00	2.18	11.12	15.65	15.85
SEM		1.77	2.68	3.97	4.96	5.00
Mechanical treatment(M)						
None		0.67	4.85	16.85	22.87	22.88
Harsh		-1.42	-0.47	3.13	7.17	7.98
Mild		3.22	5.42	11.92	18.10	18.4
SEM		2.17	3.28	4.86	6.08	6.12
I× M						
Inoculated	None	2.70	8.10	20.20	24.90	24.9
Inoculated	Harsh	-0.20	0.70	2.80	8.80	10.4
Inoculated	Mild	2.45	4.25	7.45	15.65	15.65
None	None	-1.35	1.60	13.50	20.85	20.85
None	Harsh	-2.65	-1.65	3.45	5.55	5.55
None	Mild	4.00	6.60	16.40	20.55	21.15
SEM		3.07	4.64	6.87	8.59	8.66

Mild=pounding with a metal, Harsh=mincing with mincer

Table 7. Gas volume (mL) (mean±SEM) from meadow fescue silage (n=2) during 96 h *in vitro* incubation with buffered rumen liquid. Gas from buffered rumen liquid without substrate (n=2) was used as baseline.

Treatments		Hours				
		12h	24h	48h	72h	96h
Inoculation(I)						
Inoculated		-0.55	-0.18	0.05	0.27	0.88
None		-2.47	-1.73	0.22	2.42	4.80
SEM		1.10	1.33	2.26	3.50	4.08
Mechanical treatment(M)						
None		-2.58	-2.03	-1.68	-1.33	-0.40
Harsh		-2.25	-1.98	-1.47	-0.97	1.35
Mild		0.30	1.13	3.55	6.33	7.58
SEM		1.35	1.62	2.77	4.28	5.00
I × M						
Inoculated	None	3.85	4.80	5.15	5.50	7.35
Inoculated	Harsh	-2.65	-2.5	-2.15	-1.85	-1.85
Inoculated	Mild	-2.85	-2.85	-2.85	-2.85	-2.85
None	None	-9.00	-8.85	-8.50	-8.15	-8.15
None	Harsh	-1.85	-1.45	-0.80	-0.10	4.55
None	Mild	3.45	5.10	9.95	15.50	18.00
SEM		1.91	2.30	3.92	6.06	7.07

Mild=pounding with a metal, Harsh=mincing with mincer.

5 Conclusion

Inoculation with *L. buchneri* increased silage pH but mechanical treatments reduced the silage pH. In the ryegrass trial, it seems that the inoculant dominated the fermentation when applied alone but when the inoculation was combined with harsh mechanical treatment, the inoculant was unsuccessful to dominate the fermentation. FAEI alone or along with mechanical treatment had no effect on NDFD of ryegrass and Meadow fescue silages.

Acknowledgement

This publication has been produced during my scholarship period from Swedish Institute scholarship at Swedish University of Agricultural Sciences, thanks to the Swedish Institute scholarship which covers full expense of the study. Next I would like to express my deepest gratitude to my main supervisor Kamyar Mogdiniyai Kasmaei for his endless support during this master's degree project in proposal establishment, laboratory work, data analysis and interpretation. Furthermore, he also put his print in the thesis write up by giving critical comments. Kamyar you taught me the value and meaning of research practically and opened my eye to read the environment where I live.

6 Reference

Addah, W., Baah, J., Okine, E.K. & McAllister, T.A. (2012). A third-generation esterase inoculant alters fermentation pattern and improves aerobic stability of barley silage and the efficiency of body weight gain of growing feedlot cattle1. *Journal of animal science*, vol. 90 (5), pp. 1541–1552

Allen, M.S. (1997). Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber. *Journal of dairy science*, vol. 80 (7), pp. 1447–1462

Archer, J.A., Richardson, E.C., Herd, R.M. & Arthur, P.F. (1999). Potential for selection to improve efficiency of feed use in beef cattle: a review. *Australian Journal of Agricultural Research*, vol. 50 (2), pp. 147–162

Ashbell, G., Weinberg, Z.G., Hen, Y. & Filya, I. (2002). The effects of temperature on the aerobic stability of wheat and corn silages. *Journal of Industrial Microbiology and Biotechnology*, vol. 28 (5), pp. 261–263. DOI: https://doi.org/10.1038/sj/jim/7000237

Bal, M.A., Shaver, R.D., Jirovec, A.G., Shinners, K.J. & Coors, J.G. (2000). Crop Processing and Chop Length of Corn Silage: Effects on Intake, Digestion, and Milk Production by Dairy Cows. *Journal of Dairy Science*, vol. 83 (6), pp. 1264–1273. DOI: https://doi.org/10.3168/jds.S0022-0302(00)74993-9

Beauchemin, K.A., Colombatto, D., Morgavi, D.P., Yang, W.Z. & Rode, L.M. (2004). Mode of action of exogenous cell wall degrading enzymes for ruminants. *Canadian Journal of Animal Science*, vol. 84 (1), pp. 13–22. DOI: https://doi.org/10.4141/A02-102

Beecher, M., Buckley, F., Waters, S.M., Boland, T.M., Enriquez-Hidalgo, D., Deighton, M.H., O'Donovan, M. & Lewis, E. (2014). Gastrointestinal tract size, total-tract digestibility, and rumen microflora in different dairy cow genotypes. *Journal of dairy science*, vol. 97 (6), pp. 3906–3917

Beever, D.E., Thomson, D.J., Ulyatt, M.J., Cammell, S.B. & Spooner, M.C. (1985). The digestion of fresh perennial ryegrass (Lolium perenne L. cv. Melle) and white clover (Trifolium repens L. cv. Blanca) by growing cattle fed indoors. *British Journal of Nutrition*, vol. 54 (3), pp. 763–775

Bergman, E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews*, vol. 70 (2), pp. 567–590. DOI: https://doi.org/10.1152/physrev.1990.70.2.567

Berry, D.P., Horan, B., O'Donovan, M., Buckley, F., Kennedy, E., McEvoy, M. & Dillon, P. (2007). Genetics of Grass Dry Matter Intake, Energy Balance, and Digestibility in Grazing Irish Dairy Cows. *Journal of Dairy Science*, vol. 90 (10), pp. 4835–4845. DOI: https://doi.org/10.3168/jds.2007-0116

Borreani, G. & Tabacco, E. (2010). The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. *Journal of Dairy Science*, vol. 93 (6), pp. 2620–2629

Borreani, G., Tabacco, E. & Cavallarin, L. (2007). A New Oxygen Barrier Film Reduces Aerobic Deterioration in Farm-Scale Corn Silage. *Journal of Dairy Science*, vol. 90 (10), pp. 4701–4706. DOI: https://doi.org/10.3168/jds.2007-0310

Borreani, G., Tabacco, E. & Ciotti, A. (1999). Effects of mechanical conditioning on wilting of alfalfa and Italian ryegrass for ensiling. *Agronomy journal*, vol. 91 (3), pp. 457–463

Borreani, G., Tabacco, E., Schmidt, R.J., Holmes, B.J. & Muck, R.E. (2018). Silage review: Factors affecting dry matter and quality losses in silages. *Journal of Dairy Science*, vol. 101 (5), pp. 3952–3979. DOI: https://doi.org/10.3168/jds.2017-13837

Bosch, M.W., Tamminga, S., Post, G., Leffering, C.P. & Muylaert, J.M. (1992). Influence of stage of maturity of grass silages on digestion processes in dairy cows. 1. Composition, nylon bag degradation rates, fermentation characteristics, digestibility and intake. *Livestock Production Science*, vol. 32 (3), pp. 245–264. DOI: https://doi.org/10.1016/S0301-6226(12)80005-4

Broderick, G.A., Brito, A.F. & Colmenero, J.J.O. (2007). Effects of Feeding Formate-Treated Alfalfa Silage or Red Clover Silage on the Production of Lactating Dairy Cows1. *Journal of Dairy Science*, vol. 90 (3), pp. 1378–1391. DOI: https://doi.org/10.3168/jds.S0022-0302(07)71624-7

Brüning, D., Gerlach, K., Wei\s s, K. & Südekum, K.-H. (2018). Effect of compaction, delayed sealing and aerobic exposure on forage choice and short-term intake of maize silage by goats. *Grass and forage science*, vol. 73 (2), pp. 392–405

Buxton, D.R. & Redfearn, D.D. (1997). Plant limitations to fiber digestion and utilization. *The Journal of Nutrition*, vol. 127 (5), pp. 814S–818S

Chai, W. & Udén, P. (1998). An alternative oven method combined with different detergent strengths in the analysis of neutral detergent fibre. *Animal Feed Science and Technology*, vol. 74 (4), pp. 281–288

Cherney, J.H., Cherney, D.J.R., Akin, D.E. & Axtell, J.D. (1991). Potential of Brown-Midrib, Low-Lignin Mutants for Improving Forage Quality. In: Sparks, D.L. (ed.) *Advances in Agronomy*. Academic Press, pp. 157–198.

Conaghan, P., O'Kiely, P. & O'Mara, F.P. (2012). Possibilities of increasing the residual water-soluble carbohydrate concentration and aerobic stability of low dry-matter perennial ryegrass silage through additive and cultivar use.

Grass and Forage Science, vol. 67 (2), pp. 177–198. DOI: https://doi.org/10.1111/j.1365-2494.2011.00833.x

Dehghani, M.R., Weisbjerg, M.R., Hvelplund, T. & Kristensen, N.B. (2012). Effect of enzyme addition to forage at ensiling on silage chemical composition and NDF degradation characteristics. *Livestock Science*, vol. 150 (1–3), pp. 51–58

Dohme, F., Graf, C.M., Arrigo, Y., Wyss, U. & Kreuzer, M. (2007). Effect of botanical characteristics, growth stage and method of conservation on factors related to the physical structure of forage–an attempt towards a better understanding of the effectiveness of fibre in ruminants. *Animal Feed Science and Technology*, vol. 138 (3–4), pp. 205–227

Donaghy, J., Kelly, P.F. & McKay, A.M. (1998). Detection of ferulic acid esterase production by Bacillus spp. and lactobacilli. *Applied Microbiology and Biotechnology*, vol. 50 (2), pp. 257–260. DOI: https://doi.org/10.1007/s002530051286

Driehuis, F. (2013). Silage and the safety and quality of dairy foods: a review. *Agricultural and Food Science*, vol. 22 (1), pp. 16–34. DOI: https://doi.org/10.23986/afsci.6699

Driehuis, F., Elferink, S.J.W.H.O. & Wikselaar, P.G.V. (2001). Fermentation characteristics and aerobic stability of grass silage inoculated with Lactobacillus buchneri, with or without homofermentative lactic acid bacteria. *Grass and Forage Science*, vol. 56 (4), pp. 330–343. DOI: https://doi.org/10.1046/j.1365-2494.2001.00282.x

Engels, F.M. (1987). changes in the physical/chemical structures of cell walls during growthand degradation; editted by Van Der Mear,J.M; Rijkens, B.A. Ferranti, M.P. *Degradation of lingocellulosics in ruminants and in industrial processes*. London, UK: Elsevier Applied Science Publishers LTD, pp. 13–20.

Estrada, A., Estrada, R.C., Meritt Jr, D., Bultrini, D.B., Klein, A., Dewenter, I.S., Tscharntke, T., Taniguchi, K., Wang, X. & Collomb, P. (2011). World livestock 2011: livestock in food security. *No. FAO*, vol. 363, p. W927

Finch, S., Samuel, A. & Lane, G.P. (2014). *Lockhart and Wiseman's Crop Husbandry Including Grassland*. Elsevier.

Fransen, S.C. & Strubi, F.J. (1998). Relationships among absorbents on the reduction of grass silage effluent and silage quality. *Journal of dairy science*, vol. 81 (10), pp. 2633–2644

Garcia, A.D., Olson, W.G., Otterby, D.E., Linn, J.G. & Hansen, W.P. (1989). Effects of Temperature, Moisture, and Aeration on Fermentation of Alfalfa Silage1. *Journal of Dairy Science*, vol. 72 (1), pp. 93–103. DOI: https://doi.org/10.3168/jds.S0022-0302(89)79084-6

Gardner, K.H. & Blackwell, J. (1974). The structure of native cellulose. *Biopolymers*, vol. 13 (10), pp. 1975–2001. DOI: https://doi.org/10.1002/bip.1974.360131005

Grabber, J.H. (2005). How Do Lignin Composition, Structure, and Cross-Linking Affect Degradability? A Review of Cell Wall Model Studies. *Crop Science*, vol. 45 (3), pp. 820–831. DOI: https://doi.org/10.2135/cropsci2004.0191

Gregorini, P., Soder, K.J., Sanderson, M.A. & Ziegler, G.R. (2009). Toughness, particle size and chemical composition of meadow fescue (Festuca pratensis Hud.) herbage as affected by time of day. *Animal Feed Science and Technology*, vol. 151 (3–4), pp. 330–336. DOI: https://doi.org/10.1016/j.anifeedsci.2009.02.004

Guo, G., Yuan, X., Li, L., Wen, A. & Shao, T. (2014). Effects of fibrolytic enzymes, molasses and lactic acid bacteria on fermentation quality of mixed silage of corn and hulless–barely straw in the Tibetan Plateau. *Grassland Science*, vol. 60 (4), pp. 240–246. DOI: https://doi.org/10.1111/grs.12060

Guyomard, H., Manceron, S. & Peyraud, J.-L. (2013). Trade in feed grains, animals, and animal products: Current trends, future prospects, and main issues. *Animal Frontiers*, vol. 3 (1), pp. 14–18

Heikkila, T., Saarisalo, E & Khalili, H. (2012). Effect of different chemical additives on silage quality and aerobic stability. *Proceedings of international silage conference*, Hämeenlinna, Finland, 2012. pp. 388–389. Hämeenlinna, Finland: MTT Agrifood Research Finland, University of Helsinki

Hong, B.J., Broderick, G.A., Panciera, M.P., Koegel, R.G. & Shinners, K.J. (1988). Effect of Shredding Alfalfa Stems on Fiber Digestion Determined by In Vitro Procedures and Scanning Electron Microscopy1. *Journal of Dairy Science*, vol. 71 (6), pp. 1536–1545. DOI: https://doi.org/10.3168/jds.S0022-0302(88)79717-9

Jaakkola, S., Huhtanen, P. & Vanhatalo, A. (1990). Fermentation Quality of Grass Silage Treated with Enzymes or Formic Acid and Nutritive Value in Growing Cattle Fed with or without Fish Meal. *Acta Agriculturae Scandinavica*, vol. 40 (4), pp. 403–414. DOI: https://doi.org/10.1080/00015129009438576

Jatkauskas, J., Vrotniakiene, V., Ohlsson, C. & Lund, B. (2013). The effects of three silage inoculants on aerobic stability in grass, clover-grass, lucerne and maize silages. *Agricultural and Food Science*, vol. 22 (1), pp. 137–144

Jin, L., Duniere, L., Lynch, J.P., McAllister, T.A., Baah, J. & Wang, Y. (2015). Impact of ferulic acid esterase producing lactobacilli and fibrolytic enzymes on conservation characteristics, aerobic stability and fiber degradability of barley silage. *Animal Feed Science and Technology*, vol. 207, pp. 62–74. DOI: https://doi.org/10.1016/j.anifeedsci.2015.06.011

Johnson, L.M., Harrison, J.H., Davidson, D., Mahanna, W.C. & Shinners, K. (2003). Corn Silage Management: Effects of Hybrid, Maturity, Inoculation, and Mechanical Processing on Fermentation Characteristics. *Journal of Dairy Science*, vol. 86 (1), pp. 287–308. DOI: https://doi.org/10.3168/jds.S0022-0302(03)73607-8

Jung, H.G. & Engels, F.M. (2001). Alfalfa stem tissues: rate and extent of cell-wall thinning during ruminal degradation. *NJAS - Wageningen Journal of Life Sciences*, vol. 49 (1), pp. 3–13. DOI: https://doi.org/10.1016/S1573-5214(01)80012-9

Jung, H.-J.G., Samac, D.A. & Sarath, G. (2012). Modifying crops to increase cell wall digestibility. *Plant Science: An International Journal of Experimental Plant Biology*, vols. 185–186, pp. 65–77

Kang, T.W., Adesogan, A.T., Kim, S.C. & Lee, S.S. (2009). Effects of an esterase-producing inoculant on fermentation, aerobic stability, and neutral detergent fiber digestibility of corn silage. *Journal of dairy science*, vol. 92 (2), pp. 732–738

Khota, W., Pholsen, S., Higgs, D. & Cai, Y. (2016). Natural lactic acid bacteria population of tropical grasses and their fermentation factor analysis of silage prepared with cellulase and inoculant. *Journal of Dairy Science*, vol. 99 (12), pp. 9768–9781. DOI: https://doi.org/10.3168/jds.2016-11180

Kim, S.C. & Adesogan, A.T. (2006). Influence of Ensiling Temperature, Simulated Rainfall, and Delayed Sealing on Fermentation Characteristics and Aerobic Stability of Corn Silage. *Journal of Dairy Science*, vol. 89 (8), pp. 3122–3132. DOI: https://doi.org/10.3168/jds.S0022-0302(06)72586-3

Kleinschmit, D.H. & Kung, L. (2006). The Effects of Lactobacillus buchneri 40788 and Pediococcus pentosaceus R1094 on the Fermentation of Corn Silage. *Journal of Dairy Science*, vol. 89 (10), pp. 3999–4004. DOI: https://doi.org/10.3168/jds.S0022-0302(06)72443-2

Kleinschmit, D.H., Schmidt, R.J. & Kung, L. (2005). The Effects of Various Antifungal Additives on the Fermentation and Aerobic Stability of Corn Silage. *Journal of Dairy Science*, vol. 88 (6), pp. 2130–2139. DOI: https://doi.org/10.3168/jds.S0022-0302(05)72889-7

Knicky, M. & Spörndly, R. (2011). The ensiling capability of a mixture of sodium benzoate, potassium sorbate, and sodium nitrite. *Journal of Dairy Science*, vol. 94 (2), pp. 824–831. DOI: https://doi.org/10.3168/jds.2010-3364

König, W., Lamminen, M., Weiss, K., Tuomivirta, T.T., Muñoz, S.S., Fritze, H., Elo, K., Puhakka, L., Vanhatalo, A. & Jaakkola, S. (2017). The effect of additives on the quality of white lupin–wheat silage assessed by fermentation pattern and qPCR quantification of clostridia. *Grass and Forage Science*, vol. 72 (4), pp. 757–771. DOI: https://doi.org/10.1111/gfs.12276

Kristensen, N.B., Sloth, K.H., Højberg, O., Spliid, N.H., Jensen, C. & Thøgersen, R. (2010). Effects of microbial inoculants on corn silage fermentation, microbial contents, aerobic stability, and milk production under field conditions. *Journal of Dairy Science*, vol. 93 (8), pp. 3764–3774. DOI: https://doi.org/10.3168/jds.2010-3136

Kung, L. & Ranjit, N.K. (2001). The Effect of Lactobacillus buchneri and Other Additives on the Fermentation and Aerobic Stability of Barley Silage. *Journal of Dairy Science*, vol. 84 (5), pp. 1149–1155. DOI: https://doi.org/10.3168/jds.S0022-0302(01)74575-4

Lin, K.W., Patterson, J.A. & Ladisch, M.R. (1985). Anaerobic fermentation: Microbes from ruminants. *Enzyme and Microbial Technology*, vol. 7 (3), pp. 98–107. DOI: https://doi.org/10.1016/0141-0229(85)90137-1

Liu, Q., Zhang, J., Shi, S. & Sun, Q. (2011). The effects of wilting and storage temperatures on the fermentation quality and aerobic stability of stylo silage. *Animal Science Journal*, vol. 82 (4), pp. 549–553. DOI: https://doi.org/10.1111/j.1740-0929.2011.00873.x

Lynch, J.P., Baah, J. & Beauchemin, K.A. (2015). Conservation, fiber digestibility, and nutritive value of corn harvested at 2 cutting heights and ensiled

with fibrolytic enzymes, either alone or with a ferulic acid esterase-producing inoculant. *Journal of dairy science*, vol. 98 (2), pp. 1214–1224
Lynch, J.P., Jin, L., Lara, E.C., Baah, J. & Beauchemin, K.A. (2014). The effect of exogenous fibrolytic enzymes and a ferulic acid esterase-producing inoculant on the fibre degradability, chemical composition and conservation characteristics of alfalfa silage. *Animal Feed Science and Technology*, vol. 193, pp. 21–31
McDnald, P., Edwards, R.A., GreenHalgh, J.F.D. & Morgan, C.A. (2010). *Animal nutrition*. Seventh Editon. London, UK: Pearson Education.
McDonald, P., Edwards, R.A., GreenHalgh, J.F.D. & Morgan, C.A. (2002). *Animal Nutrition*. Sixth Edition. Pearson Education.
McDonald,P., Henderson, A.R. & Heron, S:J.E (1991). *The Biochemistry of Silage*. second edition. Marlow, Bucks SL7 3PU: Chalombe publications.
Mertens, D.R. (2002). Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. *Journal of AOAC international*, vol. 85 (6), pp. 1217–1240
Mogodiniyai Kasmaei, K., Spörndly, R. & Udén, P. (2015). Separating the effects of pre-ensiled chemical and microbial composition on silage fermentation and aerobic stability. *Proceedings of Nordic Feed Science Conference*, Uppsala, 2015. Uppsala
Moore, K.J. & Jung, H.-J.G. (2001a). Lignin and Fiber Digestion. *Journal of Range Management*, vol. 54 (4), pp. 420–430. DOI: https://doi.org/10.2307/4003113
Moore, K.J. & Jung, H.-J.G. (2001b). Lignin and fiber digestion. *Journal of range management*, pp. 420–430
Muck, R.E., Nadeau, E.M.G., McAllister, T.A., Contreras-Govea, F.E., Santos, M.C. & Kung, L. (2018). Silage review: Recent advances and future uses of silage additives. *Journal of Dairy Science*, vol. 101 (5), pp. 3980–4000. DOI: https://doi.org/10.3168/jds.2017-13839
Nadeau, E.M.G., Buxton, D.R., Lindgren, E. & Lingvall, P. (1996). Kinetics of Cell-Wall Digestion of Orchardgrass and Alfalfa Silages Treated with Cellulase and Formic Acid1, 2. *Journal of Dairy Science*, vol. 79 (12), pp. 2207–2216. DOI: https://doi.org/10.3168/jds.S0022-0302(96)76597-9
Nadeau, E.M.G., Russell, J.R. & Buxton, D.R. (2000). Intake, digestibility, and composition of orchardgrass and alfalfa silages treated with cellulase, inoculant, and formic acid fed to lambs. *Journal of Animal Science*, vol. 78 (11), pp. 2980–2989. DOI: https://doi.org/10.2527/2000.78112980x
Nsereko, V.L., Smiley, B.K., Rutherford, W.M., Spielbauer, A., Forrester, K.J., Hettinger, G.H., Harman, E.K. & Harman, B.R. (2008). Influence of inoculating forage with lactic acid bacterial strains that produce ferulate esterase on ensilage and ruminal degradation of fiber. *Animal feed science and technology*, vol. 145 (1–4), pp. 122–135
Oba, M. & Allen, M.S. (1999). Evaluation of the Importance of the Digestibility of Neutral Detergent Fiber from Forage: Effects on Dry Matter Intake and Milk Yield of Dairy Cows. *Journal of Dairy Science*, vol. 82 (3), pp. 589–596. DOI: https://doi.org/10.3168/jds.S0022-0302(99)75271-9

Offical methods of the Association of Official analytical chemists (1990). *Official methods of analysis of the Association of Official Analytical Chemists*. The Association.

Ogunade, I.M., Kim, D.H., Jiang, Y., Weinberg, Z.G., Jeong, K.C. & Adesogan, A.T. (2016). Control of Escherichia coli O157:H7 in contaminated alfalfa silage: Effects of silage additives. *Journal of Dairy Science*, vol. 99 (6), pp. 4427–4436. DOI: https://doi.org/10.3168/jds.2015-10766

Oliveira, A.S., Weinberg, Z.G., Ogunade, I.M., Cervantes, A.A.P., Arriola, K.G., Jiang, Y., Kim, D., Li, X., Gonçalves, M.C.M., Vyas, D. & Adesogan, A.T. (2017). Meta-analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. *Journal of Dairy Science*, vol. 100 (6), pp. 4587–4603. DOI: https://doi.org/10.3168/jds.2016-11815

Oude Elferink, S.J.W.H., Krooneman, J., Gottschal, J.C., Spoelstra, S.F., Faber, F. & Driehuis, F. (2001). Anaerobic Conversion of Lactic Acid to Acetic Acid and 1,2-Propanediol by Lactobacillus buchneri. *Applied and Environmental Microbiology*, vol. 67 (1), pp. 125–132. DOI: https://doi.org/10.1128/AEM.67.1.125-132.2001

Pahlow, G., Muck, R.E., Driehuis, F., Oude elferink,S.J.W.H. & Spoelstra, S.F. (2003). Microbiology of ensiling. *Silage science and Technology*, pp. 31–93

Pauly (2010). Minimum temperature for the successful fermentation of corn silage. *Proceedings of Nordic feed science*, Uppsala, Sweden, 2010. pp. 103–106. Uppsala, Sweden: Swedish University of Agricultural Sciences, Department of Animal Nutrition …

Pu, Y., Hu, F., Huang, F., Davison, B.H. & Ragauskas, A.J. (2013). Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. *Biotechnology for biofuels*, vol. 6 (1), p. 15

Ralph, J., Guillaumie, S., Grabber, J.H., Lapierre, C. & Barrière, Y. (2004). Genetic and molecular basis of grass cell-wall biosynthesis and degradability. III. Towards a forage grass ideotype. *Comptes Rendus Biologies*, vol. 327 (5), pp. 467–479. DOI: https://doi.org/10.1016/j.crvi.2004.03.004

Reich, L.J. & Kung Jr, L. (2010). Effects of combining Lactobacillus buchneri 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. *Animal Feed Science and Technology*, vol. 159 (3–4), pp. 105–109

Richardson, E.C. & Herd, R.M. (2004). Biological basis for variation in residual feed intake in beef cattle. 2. Synthesis of results following divergent selection. *Australian Journal of Experimental Agriculture*, vol. 44 (5), pp. 431–440. DOI: https://doi.org/10.1071/ea02221

Rubin, E.M. (2008). Genomics of cellulosic biofuels. *Nature*, vol. 454 (7206), p. 841

Saarisalo, E., Jalava, T., Skyttä, E., Haikara, A. & Jaakkola, S. (2006). Effect of lactic acid bacteria inoculants, formic acid, potassium sorbate and sodium benzoate on fermentation quality and aerobic stability of wilted grass silage. Available at: http://jukuri.luke.fi/handle/10024/463509 [2019-06-04]

Scheller, H.V. & Ulvskov, P. (2010). Hemicelluloses. *Annual review of plant biology*, vol. 61

Seppälä, A., Heikkilä, T., Mäki, M. & Rinne, M. (2016). Effects of additives on the fermentation and aerobic stability of grass silages and total mixed rations. *Grass and Forage Science*, vol. 71 (3), pp. 458–471. DOI: https://doi.org/10.1111/gfs.12221

Shao, T., Zhang, L., Shimojo, M. & Masuda, Y. (2007). Fermentation Quality of Italian Ryegrass (Lolium multiflorum Lam.) Silages Treated with Encapsulated-glucose, Glucose, Sorbic Acid and Pre-fermented Juices. *Asian-Australasian Journal of Animal Sciences*, vol. 20 (11), pp. 1699–1704. DOI: https://doi.org/2007.20.11.1699

Silva, V.P., Pereira, O.G., Leandro, E.S., Da Silva, T.C., Ribeiro, K.G., Mantovani, H.C. & Santos, S.A. (2016). Effects of lactic acid bacteria with bacteriocinogenic potential on the fermentation profile and chemical composition of alfalfa silage in tropical conditions. *Journal of Dairy Science*, vol. 99 (3), pp. 1895–1902. DOI: https://doi.org/10.3168/jds.2015-9792

Smith, J., Sones, K., Grace, D., MacMillan, S., Tarawali, S. & Herrero, M. (2013). Beyond milk, meat, and eggs: Role of livestock in food and nutrition security. *Animal Frontiers*, vol. 3 (1), pp. 6–13. DOI: https://doi.org/10.2527/af.2013-0002

Taylor, C.C. & Kung, L. (2002). The Effect of Lactobacillus buchneri 40788 on the Fermentation and Aerobic Stability of High Moisture Corn in Laboratory Silos1. *Journal of Dairy Science*, vol. 85 (6), pp. 1526–1532. DOI: https://doi.org/10.3168/jds.S0022-0302(02)74222-7

Udén, P. (2010). The influence of sample preparation of forage crops and silages on recovery of soluble and non-structural carbohydrates and their predictions by Fourier transform mid-IR transmission spectroscopy. *Animal Feed Science and Technology*, vol. 160 (1), pp. 49–61. DOI: https://doi.org/10.1016/j.anifeedsci.2010.06.013

Van Soest (1994). *Nutritional Ecology of the Ruminant*. Cornell University Press.

Van Soest, P.J., Robertson, J.B. & Lewis, B.A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, vol. 74 (10), pp. 3583–3597. DOI: https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Vissers, M.M.M., Driehuis, F., Te Giffel, M.C., De Jong, P. & Lankveld, J.M.G. (2007). Concentrations of Butyric Acid Bacteria Spores in Silage and Relationships with Aerobic Deterioration. *Journal of Dairy Science*, vol. 90 (2), pp. 928–936. DOI: https://doi.org/10.3168/jds.S0022-0302(07)71576-X

Weinberg, Z. & Muck, R.E. (1996). New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiology Reviews*, vol. 19 (1), pp. 53–68

Weinberg, Z.G., Ashbell, G., Hen, Y. & Azrieli, A. (1993). The effect of applying lactic acid bacteria at ensiling on the aerobic stability of silages. *Journal of Applied Bacteriology*, vol. 75 (6), pp. 512–518. DOI: https://doi.org/10.1111/j.1365-2672.1993.tb01588.x

Weinberg, Z.G., Muck, R.E., Weimer, P.J., Chen, Y. & Gamburg, M. (2004). Lactic acid bacteria used in inoculants for silage as probiotics for ruminants. *Applied Biochemistry and Biotechnology*, vol. 118 (1), pp. 1–9. DOI: https://doi.org/10.1385/ABAB:118:1-3:001

Weisbjerg, M.R., Waldemar, P., Hellwing, A.L.F. & Kristensen, T. (2018). Can we increase digestibility of green forages by physical treatment before ensiling?, 2018. pp. 15–22. Swedish University of Agricultural Sciences, Department of Animal Nutrition …

Weiss, K., Kroschewski, B. & Auerbach, H. (2016). Effects of air exposure, temperature and additives on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in corn silage. *Journal of dairy science*, vol. 99 (10), pp. 8053–8069

Winters, A.L., Fychan, R. & Jones, R. (2001). Effect of formic acid and a bacterial inoculant on the amino acid composition of grass silage and on animal performance. *Grass and Forage Science*, vol. 56 (2), pp. 181–192. DOI: https://doi.org/10.1046/j.1365-2494.2001.00265.x

Yahaya, M.S., Goto, M., Yimiti, W., Smerjai, B. & Kawamoto, Y. (2004). Evaluation of fermentation quality of a tropical and temperate forage crops ensiled with additives of fermented juice of epiphytic lactic acid bacteria (FJLB). *Asian-australasian journal of animal sciences*, vol. 17 (7), pp. 942–946

Zahiroddini, H., Baah, J., Absalom, W. & McAllister, T.A. (2004). Effect of an inoculant and hydrolytic enzymes on fermentation and nutritive value of whole crop barley silage. *Animal Feed Science and Technology*, vol. 117 (3), pp. 317–330. DOI: https://doi.org/10.1016/j.anifeedsci.2004.08.013

Zhao-hai, Chao, L., Qing-fang, X., Zhu, y. & Chun-sheng, B. (2012). The effects of wilting and additive treatments on the quality of Bothriochloa ischaemum silage. *Proceedings of International silage conference*, Hämeenlinna, finland, 2012. Hämeenlinna, finland: MTT Agrifood Research Finland