MODULATIONS OF BACTERIAL COMMUNITIES OF GRASS SILAGE BY ENSILING MANAGEMENT FACTORS

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Tiivistelmä Referat Abstract

The objective was to evaluate how different silage additives can manipulate the ensiling process and the profile of bacterial communities of grass silages under varying management conditions. Silages were made from mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) grass to laboratory scale silos using two compaction levels. The tightly compacted grass was also contaminated with soil and dairy cow faeces. Four additive treatments were used including control without additive (CONT), formic acid based additive (FA), homofermentative strains of lactic acid bacteria (LAB) and salt based additive (SALT).

Tight compaction resulted on average in lower pH and ethanol concentration in silages than loose compaction mostly caused by changes in CONT silages. Soil contamination clearly affected CONT and SALT silages by stimulating extensive fermentation and thus decreasing pH and amount of residual water-soluble carbohydrates (WSC) compared to non-contaminated silages. In all conditions, FA restricted fermentation resulting in silages with high WSC and reduced total fermentation products concentration. Soil contamination improved aerobic stability of silages compared to non-contaminated ones because of higher acetic acid concentration in contaminated silages.

Abundance of selected 16 bacteria in raw material was low, with *Sphingomonas* and *Stenotrophomonas* genera being the most abundant. After fermentation both *Lactobacillaceae* family and as part of it *Lactobacillus* genus were dominant with *Sphingomonas* genus in most of the silages. FA decreased the abundance of *Lactobacillaceae* family whereas LAB increased it. Soil contamination reduced the amount of other *Lactobacillaceae* family but boosted the growth of *Lactobacillus* genus. *Lactobacillus* presented negative correlations with *Mycoplana*, *Devosia* and *Sphingomonas*. Five bacteria were connected to desirable fermentation pattern and they all were part of same phylum *Firmicute*. All other selected bacteria had negative correlation with low pH and amount of lactic and total fermentation acids in silage.

Use of additives improved fermentation quality of silages ensiled under different management conditions. Different types of additives resulted in varied bacterial profiles. Results confirmed the importance of tight compaction and good hygiene for stable fermentation. Strong correlations between bacterial communities and fermentation quality parameters provided clear insight of the role of the most abundant populations on the fermentation process of grass silage.

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silage, DNA extraction, fermentation, microbiome, metagenome, *Phleum pratense*, silage additive

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Tiivistelmä Referat Abstract

Tutkimuksessa selvitettiin, kuinka erilaiset säilöntäaineet vaikuttavat rehun käymiseen ja sen bakteerikoostumukseen vaihtelevissa säilöntäolosuhteissa. Timotein (*Phleum pratense*) ja nurminadan (*Festuca pratensis*) seosnurmelle tehtiin neljä säilöntäainekäsittelyä, jotka olivat kontrolli ilman säilöntäainetta (CONT), muurahaishappoon perustuva säilöntäaine (FA), homofermentatiivinen maitohappobakteeri (LAB) ja suolapohjainen säilöntäaine (SALT). Rehu säilöttiin koesiiloihin käyttäen kahta eri tiiviystasoa. Tiiviille rehulle tehtiin myös likasaastumista kuvaava käsittely, jossa maata ja lantaa lisättiin rehuun.

Tiivis rehu johti keskimäärin alhaisempaan pH:hon ja etanolipitoisuuteen kuin löyhästi pakattu rehu, sillä erityisesti CONT rehujen koostumus vaihteli. Likasaastuminen stimuloi voimakasta käymistä CONT ja SALT rehuissa, joten niiden pH ja jäännössokerin määrä olivat alhaisempia kuin vastaavilla ei-saastuneilla rehuilla. Likasaastuneet rehut pysyivät keskimäärin pisimpään aerobisesti stabiileina, sillä erityisesti likasaastuneisiin CONT ja SALT rehuihin kertyi etikkahappoa. Läpi käsittelyjen FA kykeni rajoittamaan käymistä, säilömään rehun sokeria ja vähentämään kokonaiskäymistuotteiden määrää rehuissa.

Valittujen 16 bakteeriryhmän osuus nurmen bakteereista oli pieni ja eniten havaittiin *Sphingomonasia* ja *Stenotrophomonasia*. Käymisen jälkeen valitut ryhmät muodostivat valtaosan bakteerikannasta ja hallitsevimpia olivat *Lactobacillaceae*- heimo ja osana sitä *Lactobacillus*- suku yhdessä *Sphingomonasin* kanssa. Suhteellisesti FA pienensi *Lactobacillaceae*- heimon osuutta havainnoista, kun taas osuus oli luonnollisesti suurin LAB rehuissa. Likasaastuminen vähensi *Lactobacillaceae*- heimon osuutta havainnoista, mutta kasvatti *Lactobacillus*- suvun osuutta. Bakteerien välillä havaittiin, että *Lactobacillus* korreloi negatiivisesti *Mycoplana*, *Devosia* ja *Sphingomonas*-sukujen kanssa. Maitohapon määrä korreloi negatiivisesti *Devosian*, *Agrobacteriumin* ja *Sphingomonasin* kanssa, ja nämä samat bakteeriryhmät olivat positiivisesti yhteydessä jäännössokerin määrän kanssa. Viiden bakteeriryhmän havaittiin olevan yhteydessä säilöntälaadun kannalta hyödylliseen käymiseen, loput johtivat lähinnä ei-toivottuun käymiseen. Viisi hyödyllistä bakteeria olivat ainoat *Firmikuutti*- pääjaksoon kuuluvat bakteerit tässä tutkimuksessa.

Säilöntäainekäsittelyn havaittiin parantavan rehun säilöntälaatua vaihtelevissa säilöntäoloissa. Eri säilöntäaineet johtivat erityyppisiin mikrobiomeihin ja bakteerikoostumuksiin. Havaitut korrelaatiot bakteeriryhmien ja käymisparametrien välillä kertoivat, kuinka tietyt dominoivat bakteeriryhmät vaikuttavat käymistapahtumaan.

Avainsanat Nyckelord Keywords
säilörehu, DNA:n eristäminen, käyminen, mikrobiomi, metagenomi, *Phleum pratense*, säilöntäaine

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1 Abbreviations

CONT	Control treatment
CFU	Colony forming units
CP	Crude protein
DM	Dry matter
DNA	Deoxyribonucleic acid
FM	Fresh matter
FA	Formic acid based additive treatment
LAB	Lactic acid bacteria based treatment
NDF	Neutral detergent fibre
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SALT	Salt based additive treatment
VFA	Volatile fatty acids
WSC	Water soluble carbohydrates

2 Introduction

Milk production is based on ensiled forages especially in climate conditions where grazing is limited. Grass crops have a great potential to produce biomass and valuable animal feed from solar energy with photosynthesis (Hopkins 2000). In 2019, there were 783 800 hectares of grass fields in Finland, which was 34 % of total agricultural area (Luke 2019). Over the last decades the proportion of silage as a feed for dairy cows has increased worldwide while usage of pastures and hay has decreased, because more intensive feeding enables higher milk yields than grazing and use of dry hay to dairy cows (Wilkinson and Rinne 2018).

Successful silage production plays important role in dairy farm's economy. Success in ensiling depends on five main topics: forage crop, harvest time management, silo type, silo management and silage additives (Muck and Kung 2007). Ensiling process has major impacts to silage intake, milk production and animal welfare. Poor fermentation quality increases losses in silage and decreases nutritional quality of silage, which is evaluated based on nutritional needs of animal (Harrison et al. 2003). Unwanted fermentation products such as high amounts of volatile fatty acids (VFA) and total fermentation acids decreases the voluntary feed intake of dairy cows (Huhtanen et al. 2007).

Preservation of moist plant material is possible because of anaerobic fermentation process which decreases pH in silage, ceases the plant respiration and proteolysis and inhibits the growth of harmful microbes (McDonald et al. 1991 p.11-12). Anaerobic conditions are essential so that aerobic microbes are not able to grow in silage and spoil it.

Fresh plant material contains large variety of epiphytic microbes like bacteria, yeasts and moulds. Silage fermentation is a result of the activity of these microbial communities. They start the fermentation process naturally, but different kinds of additives are used to control, restrict or stimulate the fermentation. Wild type of fermentation is unpredictable, and it can lead to ensiling losses and increase the amount of harmful fermentation end products and ammonia in silages.

Additives cannot fully compensate poor management during harvesting and preserving but they can decrease the risk of poor fermentation for example in unfavourable conditions. It is important to maintain good hygiene during silage making, compact the silage and cover the silo, clamp or bale with plastic as fast as possible.

Evaluating the diversity of microbial communities in different raw materials and in fermented silages is important so that we would be able to control ensiling process with additives and management factors and understand the microbial shifts during fermentation. Each bacterial community is able to affect the direction of fermentation and the final quality of silage. For a long time, it was difficult and expensive to identify all microbial communities in silage, but nowadays DNA extraction and sequencing techniques are more efficient and cost-effective than previously (Heather and Chain 2016).

3 Ensiling and microbial communities

3.1. Ensiling process

In Finland the most common crop is timothy (*Phleum pretense*) mixed with other plants such as meadow fescue (*Festuca pratensis*), tall fescue (*Festuca arundinacea)* and/or red clover (*Trifolium pretense*). In warmer climates, maize (*Zea mays L.*) and different ryegrass (*Lolium*) species are common choices for silage and those crops have typically higher concentration of water soluble carbohydrates (WSC) than timothy and fescues (McDonald et al. 1991 p.19).

Ensiling is a forage preservation method based on lactic acid fermentation in anaerobic circumstances. Mown or chopped plant material is stored in silo, clamp, tower or bale and sealed off to create anaerobic storage (McDonald et al. 1991 p.12-14). Pre-wilting the plant material on a field before chopping is a common way to increase the dry matter (DM) content.

Ensiling process can be divided into four phases. The 1st phase is aerobic and starts when forage is cut and ends when all the air is consumed after sealing the silo (Piltz and Kaiser 2004). Plant respiration, enzymes and aerobic

microbes utilize all oxygen in the silo within a few hours after sealing, but if the silo is poorly packed, plant respiration and enzyme activity can continue for even some days (Kung 2014). One of the main reasons for good compaction is to push most of the air out of the silage and fit as much material to silo as possible. Proteolysis is stronger in very moist silage than in high DM silage (McDonald et al. 1991 p.62). Plant respiration, proteolysis by enzymes and activity of harmful microbes induce losses of protein, energy, DM and carbohydrates in silage, so it is important to abbreviate the length of this phase (Piltz and Kaiser 2004).

The 2nd fermentation phase starts when all the oxygen is consumed out in silo. Anaerobic lactic acid bacteria start to dominate the fermentation. They utilize water-soluble carbohydrates to produce lactic acid which decreases the pH in silage. The fast drop of pH is important to inhibit the growth of harmful microbes such as Enterobacteria and Clostridia. According to Kung (2014) fermentation is controlled by the type of dominating bacteria, amount of substrate and moisture content of the silage. Main fermentation phase takes time from one week to a month and silo should not be opened before this phase has ended (Merry et al. 2000).

After the main fermentation phase, the pH has dropped to near 4.0 depending on dominant bacteria and DM of silage. In this pH even most of the lactic acid bacteria stop growing. In this 3rd phase silage is stable for a long time if anaerobic circumstance is maintained. In stable silage, losses can stay minimal for several years after ensiling.

The 4th phase starts when silo is opened and exposed to oxygen again. Good quality silage remains stable during normal feed-out time but if there has been some unwanted fermentation, silage can start to spoil, change its composition and heat after only a few hours (Kung 2014). In this phase, yeasts, moulds and aerobic undesirable bacteria, which have survived as dormant spores, start to grow and produce metabolic products and cause losses in nutritional value and DM. Silage pH often increases a little because microbial activity starts to break down fermentation acids (Rooke and Hatfield 2003). Aerobic stability of silage in feed-out phase is a result of harvesting, silo filling and fermentation management factors (Wilkinson and Davies 2012). Heterofermentative lactic

acid bacteria inoculants produce some ethanol, acetic acid or 1,2-propanediol to increases aerobic stability, but they can also increase energy and DM losses in silage. Other additives, such as propionic acid and benzoic acid contain substances that can delay heating.

3.2 Bacterial communities of raw material and silage

Rich variety of bacterial species is present in surfaces of plant material as epiphytic bacteria. Most of these bacteria are aerobic species which either die or move to dormant mode as endospores in anaerobic conditions. The number of epiphytic bacteria in raw material varies by the maturity of crop, wilting and plant species and it is always higher in leaves than in stems (McDonald et al. 1991 p.81). Numbers of aerobic *Pseudomonas* and *Enterobacteria* can reach $1×10^7$ colony forming units (CFU)/g of fresh forage (Pahlow 1991) and according to Pahlow et al. (2003) epiphytic lactic acid bacteria can variate between $10 - 10^6$ CFU/g FM (Table 1). This means that lactic acid bacteria are not usually dominant in epiphytic bacteria. Natural microflora in plant material increases rapidly after cutting if wilting last more than six hours (Lin et al. 1992).

Table 1. Typical populations of bacterial and fungal groups on plants prior to ensiling (Pahlow et al. 2003).

Group	Population (colony-forming units/g crop)	Log10
Total aerobic bacteria	>10 000 000	>7
Lactic acid bacteria	10 – 1 000 000	1-6
Enterobacteria	1000 – 1 000 000	3-6
Yeasts and yeast-like fungi	1000 – 100 000	3-5
Moulds	1000 – 10 000	3-4
Clostridia (endospores)	100 - 1000	2-3
Bacilli (endospores)	100 - 1000	2-3
Acetic acid bacteria	100 - 1000	2-3
Propionic acid bacteria	10 - 1000	1-3

After sealing when all the air is consumed, microbes capable of anaerobic growth continue or begin to grow and use all kinds of available nutrients. McDonald et al. (1991, p.81) mentions that lactic acid bacteria, *Enterobacteria*, *Clostridia*, some *Bacillus* species and some yeasts are active in early phase of fermentation. Bacterial communities interact with each other in many ways: competing for same available nutrients, colonizing extra space or boosting the growth of other species (Eikmeyer et al. 2013, Stubbendieck et al. 2016). Merry et al. (2000) mentioned that *Pediococcus pentosaceus* together with *Lactobacillus plantarum* were more efficient to lower pH of silage during first days of fermentation than *Lactobacillus plantarum* alone. *Pediococcus* produces lactic acid faster in high pH silage and *Lactobacillus* is more efficient when pH declines.

Each bacterium has its own demands for surrounding circumstances to be able to grow. Typically, bacteria species have minimum, optimum and maximum for growing temperature and pH. Most of the lactic acid bacteria are mesophilic, meaning that they tolerate temperatures 5 °C – 55 °C and their optimum is around 37 °C (Wood et al. 1995). Lactic acid bacteria can tolerate low pH (even below 4.0) and that gives them advantages against other bacteria. Bacteria have variable demands for substrates such as WSC.

Some bacteria within microbiome are difficult to cultivate in laboratory, and some of *Lactobacillus* and *Lactococcus* species move easily to viable but non-culturable state (Oliver 2005, Eikmeyer et al. 2013). Therefore, cultivation-based experiments like agar plates are not able to identify those species or the number of bacteria in the sample. Agar plates lack diversity of natural environment which are often porous and physically and chemically complex surroundings (Coyte et al. 2016). Experiments based on DNA or RNA extraction allow us to identify dominant species in silage precisely and to measure the shift of bacterial communities during ensiling (McGarvey et al. 2013). High resolution melt analysis has also been used to identify bacteria species during ensiling without DNA sequencing based on increasing temperature and melting curves of bacteria (Ogiy et al. 2015).

These new technologies related to whole comparative genomic, metagenomic and metatranscriptomic can give us far more detailed information about

microbiota of harvested forage and silage than classic microbiology (McAllister et al. 2018). This information could help us to characterize the role of silage additives and epiphytic bacteria in ensiling process and aim for better silage quality as well as inhibit the abundance of yeasts, moulds and unwanted bacteria in silage.

Few studies have reported bacterial populations of different silages based on DNA/RNA sequencing. McGarvey et al. (2013) and Ogunade et al. (2018) focused on lucerne silages. McGarvey et al. (2013) found genera *Erwinia*, *Escherichia*, *Pseudomonas*, *Pantoea* and *Enterobacter* to be dominant in lucerne forage before ensiling. After fermentation process heterofermentative *Lactobacillus buchneri* was the most dominant species but homofermentative *Pediococcus pentosaceus*, *Lactobacillus garvieae* and *Lactobacillus plantarum* were also abundant with high numbers. Ogunade et al. (2018) tested the effect of *Lactobacillus plantarum*, *Lactobacillus buchneri* and propionic acid with *Escheria coli* inoculant in lucerne. They found that genera *Lactobacillus*, *Pediococcus* and *Weisella* were dominant in most of the silages. *Lactobacillus plantarum* decreased the abundance of *Pediococcus* and increased abundance of *Pantoea* compared to *Lactobacillus buchneri* treatment. With propionic acid additive the abundance of *Lactobacillus* was significantly lower than in other treatments and control.

Eikmeyer et al. (2013) examined the effect of *Lactobacillus buchneri* inoculation on bacterial community of grass silage. *Lactobacillus buchneri* was present in only those samples that were inoculated. *Lactobacillus plantarum* and *Lactobacillus brevis* were present in epiphytic flora and they had great effect to ensiling process. Eikmeyer et al. (2013) concluded that genera *Lactobacillus*, *Lactococcus*, *Weisella* and *Leuconostoc* were the main bacteria in silage samples in both after 14 and 58 days of ensiling.

Recently Xu et al. (2019) explored both bacterial communities and metabolome of corn silage. They added homo- or heterofermentative bacteria (*Lactobacillus plantarum* and *Lactobacillus buchneri*) to forage and noticed that those strains were not the most dominant during ensiling, but still they modulated bacterial communities and metabolome in many ways. Variations between bacterial communities and metabolites formed complex patterns, but some interactions to

metabolites and later to fermentation quality were seen. For example, positive correlations between bacteria *Lactobacillus silagei*, *Lactobacillus parafarraginis* and *Lactobacillus buchneri* and three essential amino acids (lysine, methionine and phenylalanine) were found. In future there is hope that information about microbiome and metabolome of silages could be used to provide better fermentation quality and to make functional silages to increase animal health and welfare. However, currently there is no such practical application available.

3.3 Factors affecting the fermentation quality

The goal of ensiling is a good fermentation quality which includes low pH of silage and low concentrations of VFAs, ethanol and ammonia N (Seglar 2003). The amount of fermentation end products and changes in silage composition reveals which kind of fermentation had happened in silage.

Typically amount of VFAs such as acetic, propionic and butyric acids are measured. Sometimes higher VFAs (valeric, isovaleric and caproic acids) are measured to get more detailed result. Amount of lactic acid is a good indicator of lactic acid fermentation in silage. Moderate amount of lactic acid is wanted to silage for good fermentation (Seglar 2003), but high amount of fermentation acids and VFAs decreases DM intake of high producing dairy cows (Huhtanen et al. 2007). Sometimes minor levels of acetic and propionic acid or ethanol are useful for silage quality because of their antimicrobial ability after opening the silo (Oude Elferink et al. 2001).

Final pH of silage should be around 4, but target pH is related to DM of silage (McDonald et al. 1991 p.12). In dry silages higher pH is tolerated than in moist silages. Low pH prevents protein degradation and nutrient losses and inhibits the growth of undesirable microbes. Usually stable pH is evidence of stable silage.

Amount of ammonia N of total N (g/kg) is a good indicator of protein degradation in silage caused by poor fermentation (Rinne and Franco 2019). Crude protein (CP), DM and WSC content can differ from results of forage before ensiling and reflect different losses in silage. Many losses are related to oxygen content in silage and therefore it is important to prevent air exposure to

silage during ensiling. Losses of WSC are related to the rate of fermentation but some preserved WSC are recommended (Korhonen 2012).

Yeasts and moulds create different losses and spoil silage. Mycotoxins such as deoxynivalenol and zearalenone are secondary metabolites of fungi, produced in environment with high temperature, humidity and oxygen (Ogunade et al. 2018). Mycotoxins are harmful for animals (McDonald et al. 1991 p.129).

Anaerobic conditions are essential for a good fermentation. It is very important to prevent exposure to air during storage time. If silo is exposed to air, yeasts start to metabolize lactic acid in silage which leads to increase of silage pH and activation of other harmful microbes (Kung 2001). Microbial activity in silo leads to spoiling and heating in silage.

During feed-out phase silage is exposed to air. Good management helps to prevent it from spoiling and heating. The removal of silage should be fast and precise to minimize the area and time that surface of silage is exposed to air. The small amount of acetic acid, propionic acid or ethanol in silage inhibits the growth of microbes such as yeast and moulds after opening the silo (Oude Elferink et al. 2001, Danner et al. 2003).

Silage additives are divided into four groups: fermentation stimulants (lactic acid bacteria, enzymes), fermentation inhibitors (acids, salts), antimicrobial additives and nutrient additives (ammonia, urea) (Kung et al. 2003).

Organic acids decrease the pH of forage quickly near or even below 4.0. Virtanen (1933) noticed that this prevented the breakdown of protein and most of undesirable microbes did not survive. In low DM forages lower pH is needed to stabilize the silage than with high DM forages. Formic acid additives are still the most popular way to preserve forage in Finland. In 2016, 78 % of silages made in silos were treated with organic acids based on data from Artturi®-analysis (Saarinen 2016).

Organic acids such as formic, propionic and sorbic acids restrict the fermentation process at the beginning by decreasing the pH quickly to pH 4-5 depending on DM, so that only lactic acid bacteria are able to grow, and later low pH restricts even lactic acid bacteria. Restricted fermentation decreases the amount of fermentation end-products like VFAs and preserves most of the WSC

in silage, which has proven to increase DM intake and lead to high protein and milk yields (Jaakkola et al. 2006). Formic acid has some antimicrobial effects against *Clostridia* (Woolford 1978). On the same time organic acids are corrosive and irritating to users and machines. Acids break cell walls in plant material and thus lead to effluent losses during compaction and ensiling of wet silages.

Lactic acid bacteria produce lactic acid as their metabolic product thus lowering the silage pH. There are several species and strains within same species, which are used as an additive. The most popular homofermentative species are *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Pediococcus acidilactici* and *Enterococcus faecium*. A few heterofermentative species such as *Lactobacillus buchneri* are also used because of the production of acetic and propionic acid, which inhibit the growth of spoilage moulds and yeasts (Oude Elferink et al. 2001, Filya 2003).

Homofermentative pathway produces lactic acid from glucose molecule:

$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH + 197\ kJ$

Heterofermentative pathways produce lactic acid and ethanol or acetic acid but also carbon dioxide and water which are not positive for the preservation:

$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2 + H_2O$

$3\ C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + CH_3COOH + 2\ C_6H_{14}O_6 + CO_2 + H_2O$

Homofermentative pathway (glycolysis) starts when WSC such as glucose and fructose are transformed first to pyruvate and later to lactate. End products are lactic acid and energy in ATP-form. Heterofermentative pathways such as bifidus pathway and 6P-gluconate pathway use WSC to produce lactic acid, acetic acid, ethanol, carbon dioxide and ATP. Homofermentative fermentation path is more efficient than heterofermentative paths (Table 2), and it produces lactic acid in a very fast rate with minimal DM and energy losses (Merry et al. 2000, Kung 2001). Both yeast and *Clostridia* fermentation pathways have remarkable losses in DM and Clostridia bacteria also in energy (Kung 2001).

Table 2. Predominant fermentation pathways in silage (Kung 2001).

Type of fermentation	Type of WSC used	End-products	Theoretical DM recovery, %	Theoretical Energy Recovery, %
Homolactic	glucose	lactic acid	100	99
Heterolactic	glucose	lactic acid, ethanol, CO_2	76	98
Heterolactic	fructose	lactic acid, acetate, CO_2, mannitol	95	99
Yeast	glucose	ethanol, CO_2	51	99
Clostridia	glucose & lactate	butyric acid, CO_2	49	82

WSC: water soluble carbohydrate, DM: Dry matter

Many bacteria are capable to adapt in different ways to metabolize WSC to pyruvates if surrounding circumstances are changing. Facultative bacteria are able to use both homolactic and heterolactic fermentations but usually they prefer one type of fermentation. Some lactic acid bacteria are able to decrease pH even lower than organic acids in normal dosage. Extremely acid feed with pH 3.5 signifies fermentation of acid tolerant bacteria instead of lactic acid bacteria and it reduces palatability of silage (Van Soest 1994).

Mixtures of many bacteria species are used to achieve all different abilities in same additive, such as fast decline of pH, ability to survive in low pH, use different WSC and prevent microbial activity after opening the silo. Many companies have their own strains of specific lactic acid bacteria and for example within *Lactobacillus plantarum* even the size of genome can vary, and strains are still the same species (Beck et al. 2019).

The salts of formic acid such as calcium and sodium formates, sometimes with sodium nitrite, have been regarded as most effective salt-based additives (McDonald et al. 1991 p.203). The effect is similar than with organic acid additives but often decline of pH is slower. At the same time salts are not as corrosive as organic acids. Some salts such as nitrites, sorbates, benzoates, propionates and acetates are used in salt-based additives because they release acids to silage and inhibit yeasts and moulds thus improving the aerobic stability

of silage (Auerbach et al. 2012, König et al. 2017). Nadeau et al. (2013) noticed that DM losses and amount of ethanol decreased, most of the yeasts died and aerobic stability increased in grass-clover silage when additive with nitrite, hexamine, sorbate, benzoate, and propionate was used compared to control. Dosage was 2 l/ton and typically dosage of salt-based additives is lower than with acids.

Sodium chloride is traditionally used in food preservation but results in feed ensiling were not convincing (Woolford 1978). Formaldehyde has been used because it prevents proteolysis and growth of harmful microbes, but later formaldehyde was proven to be a health risk for humans and use of it ended (McDonald et al. 1991 p.206-207).

The main goals of using enzymes as silage additives is to degrade cellulose and hemicellulose to WSC (McDonald et al. 1991 p.194). Lactic acid bacteria can utilize this extra amount of WSC and produce even more lactic acid than without enzymes, so enzymes and lactic acid bacteria are often used together. Degradable cell wall fraction is usually the easiest fibre to digest in rumen so overall organic matter digestibility of silage rarely increases (Jaakkola 1990, Jacobs and McAllan 1991). Sometimes some WSC are added to silage to boost the lactic acid fermentation. Different kinds of molasses have been used as additives, but they are not very effective in reasonable dosage levels (Heron et al. 1988).

Adequate compaction level and rapid silo packing are essential for good quality silage (Kung et al. 2003). Low packing densities leave air in the plant material and can lead to DM losses and increase the amount of ammonia-N in silage (Ruppel et al. 1995). A good compaction can be achieved with heavy packing tractor and putting only thin layers of forage to silos at time. According to Kung et al. (2003) grasses have hollow and hard structure of stems and thus tend to be more challenging to pack well than maize and lucerne forages. In grass silage minimum density of 210 kg DM/m^3 is recommended at the time of feed-out (Wilkinson and Davies 2012). Silage is often less packed in the edges of bunker silo or clamp, because those areas are more difficult to pack with a tractor.

Good hygiene during silage making is an important factor for successful ensiling. Contamination can increase the number of harmful bacteria in forage. Contamination can be from many sources such as soil, manure, old spoiled forage or machinery. Laws et al. (2002) studied the effects of slurry application on silage quality and found that the most significant contamination risk for ensiling is *Clostridia* when slurry is applied. *Clostridia* bacteria cause undesirable fermentation in silage and produce butyric acid. Those bacteria can metabolize lactic acid to butyric acid ($C_4H_8O_2$) and carbon dioxide. This leads to an increase in silage pH and ensiling losses and spores of *Clostridia* bacteria decreases the quality of milk products, especially in cheese production (McDonald et al. 1991 p.110, Oude Elferink et al. 2001).

Some strains of *Clostridia*, *Enterobacteria and Bacilli* can reduce nitrate in silage, first to nitrite and later to nitrous oxygen and ammonia. This increases buffering capacity and makes those bacteria more competitive against lactic acid bacteria (McDonald et al. 1991 p.105-108).

Silage particle length is important for feed intake, because cow needs good size particles for rumen activation, but they can also sort too long particles out. Cut length, particle size and crushing of plants can affect to fermentation process in silo as well. Typically, precision forage harvesters make a homogenous and short particle size whereas baling and self-loading wagons have more variation in particle length. Shredding and short particle size decrease activity of *Clostridia* and increase lactic acid production in silage (Pauly and Lingvall 1999).

3.4 Chemical composition of forage crops

A good silage crop should have adequate concentration of WSC for fermentation, DM content above 200 g/kg FM and low buffering capacity against changes in pH (McDonald et al. 1991 p.19). Especially the amount of WSC variates between crops and many silage crops used in Finland have relatively low concentration of WSC.

Table 3. Water soluble carbohydrate contents (g/kg DM) of five grass species (McDonald et al. 1991 p.25).

Species	Number of samples	Range	Mean	s.e.
Italian ryegrass	38	74-314	181	± 9.6
Perennial ryegrass	191	46-315	170	± 3.8
Timothy	20	53-199	110	± 10.7
Meadow fescue	17	35-263	96	± 14.4
Cocksfoot	107	5-191	79	± 4.1

Henderson examined the WSC content of five grass species (McDonald et al. 1991 p.25), and within those species' Italian ryegrass (*Lolium multiflorum*) had the highest and Cockfoot (*Dactylis glomerata*) the lowest amount of WSC (Table 3). Besides crop species, also cultivar, stage of growth, weather, climate and time of the day affect the content of WSC in forage (McDonald et al. 1991 p.23-27).

When silage harvesting is postponed, grass becomes more mature and its neutral detergent fibre (NDF) content increase whereas CP content and organic matter digestibility decrease (Rinne et al. 1997). High organic matter digestibility is important for good milk production, so it is recommended to harvest grass in early maturity state. Harvesting on too early maturity state on the other hand increases the amount of N in silage and leads presumably to bigger N losses from the animals. Successful fermentation does not typically affect the amount of CP in silage whereas degradation of CP to NH_3-N indicates poor fermentation (Rinne and Franco 2019).

Nitrogen fertilization increases the nitrate content of forage which can affect to fermentation quality of silage. Intermediates and end-products of nitrate (NO_3^-) degrading can improve the fermentation quality of silage. Intermediates such as nitrite (NO_2^-) and nitric oxide (NO) can inhibit *Clostridia* activity temporarily during accumulation of those compounds (Spoelstra 1985, Knický 2005). Large amount of nitrate can be toxic to animals, but nitrates of forage degrade in ensiling process.

Dry matter content of forage has remarkable effect on silage fermentation. If DM content of silage decreases to 200 g/kg FM, it increases the water activity, amount of silage effluent and nutrient losses (McDonald et al. 1991 p.167,172, Merry et al. 2000).

Buffering capacity is important factor which variates for crop to crop. Buffering capacity can be compared as an amount of acid needed for change in pH (McDonald et al. 1991 p.32). Higher buffering capacity of legumes such as clovers, lucerne and lupines increases the risk of undesirable fermentation because of slow decline of pH (Kung et al. 2003, Piltz and Kaiser 2004, Huhtanen et al. 2013). Thus, it is important to notice the legume content of forage and adjust the dosage of additive for that. Piltz and Kaiser (2004) listed buffering capacities of various forages (meq/kg DM): italian ryegrass 265-589, red clover 491-617 and oats in immature vegetative stage 732-779.

Plant species have different buffering capacity to resist changes in pH. Legumes, such as clovers, lucerne and lupines are widely used, but those have higher buffering capacity compared to grass species. Red clover has lower protein degradability in silo and in rumen compared to other forage crops, because polyphenol oxidase forms bonds between phenols and protein (Sullivan and Hatfield 2006, Vanhatalo et al. 2009, Rinne and Franco 2019).

4 Objectives of the research

This thesis is a part of the Silage Metagenomics project by Natural Resources Institute Finland (Luke).

The main objectives of this research were to screen bacterial communities in forage and to see the shift of bacterial flora from grass to final fermented silage. Experiment examined how different management factors during ensiling affect the quality of silage and variety of bacterial communities in grass. Management factors were different silage additives, compaction level and hygiene of preserving process, manipulated with soil and slurry contamination. The aim was to find bacteria communities with positive impact to silage quality as well as bacterial communities which are related to undesirable fermentation and poor quality silage.

Research hypothesis of this study were:

1. Bacterial communities are different before and after ensiling process, especially lactic acid bacteria additive shifts the proportion of bacteria species.
2. Different management factors during ensiling process result in variable fermentation patterns and also different bacterial communities profile
3. Loose compaction, preserving without additives and contamination inoculant cause more unstable and poor-quality silage than good management factors during harvesting and ensiling.
4. Some bacterial communities have negative correlation for abundance of other bacteria because they compete for same resources; some have positive effect for each other's growth.

5 Materials and methods

5.1 Forage harvesting and treatments

Mixed timothy (*Phleum pretense*) and meadow fescue (*Festuca pratensis*) grass was harvested on June 4th, 2018 at Jokioinen, Finland (60°48'N, 23°29'E) from a four years old grass. Grass was cut with mower (JF GMS 3200 Topflex, JF-Fabriken- J Freudendahl A/S, Sonderborg, Denmark and Krone EC 32 CV in front hitch, Maschinefabrik Bernard Krone GmbH, Spelle, Germany) and harvested immediately after mowing without wilting. Grass was precision chopped using farm scale forage harvester (JF FCT 1350, JF-Fabriken- J Freudendahl A/S, Sonderborg, Denmark) and transported to laboratory at Jokioinen without adding any additives during harvesting.

The experiment comprised totally 12 different treatments including three types of ensiling treatment and four additive treatments (Figure 1). Three different type of ensiling treatments were: loose compaction, tight compaction and tight compaction with soil+feaces inoculant. Loose compaction was done manually by dropping an 8 kg lead plummet to the silage in plastic silo two times after every handful of grass and tight compaction by dropping the same plummet ten times.

In order to challenge the effect of additives on fermentation quality of a low-hygienic quality raw material, contamination with soil from slurry-treated area was conducted for the tight compacted silos. Soil was collected from field in Jokioinen, two days after the area was treated with dairy cow slurry. Additional fresh cow faeces was also added to the contaminant. Soil, faeces and water supernatant was mixed 24 hours before inoculation in a formic weight basis proportion of 1:1:7, respectively. Supernatant was strongly mixed and used instead of water when additives were prepared.

Within each ensiling treatment there were four different additive treatments (Table 4):

1. Control (CONT): treatment without additive
2. Formic acid based additive (FA) at 5 litres/ton of FM (AIV Ässä Na, Eastman Chemical Company, Oulu, Finland)
3. Homofermentative lactic acid bacteria (LAB) *Lactobacillus plantarum* at 1 g/ton of FM (KOFASIL® LAC, ADDCON, Bitterfeld-Wolfen, Germany)
4. Salt based additive (SALT) at 2 litres/ton of FM (Safesil Challenge, Salinity AB, Göteborg, Sweden)

Table 4. Additives used in the experiment.

Abbr.	Company	Composition	Commercial name	Dosage
FA	Eastman Chemical Company	Formic acid Propionic acid Potassium sorbate Sodium formate	AIV Ässä Na	5 l/t Fresh matter (FM)
LAB	ADDCON	*Lactobacillus plantarum* DSM 3676 *Lactobacillus plantarum* DSM 3677 (min 1 x 10^{11} CFU/g)	KOFASIL® LAC	1 g/t FM, 1×10^5 CFU/g FM
SALT	Salinity AB	Sodium nitrite E 250 Sodium benzoate Potassium sorbate	Safesil Challenge	2 l/t FM

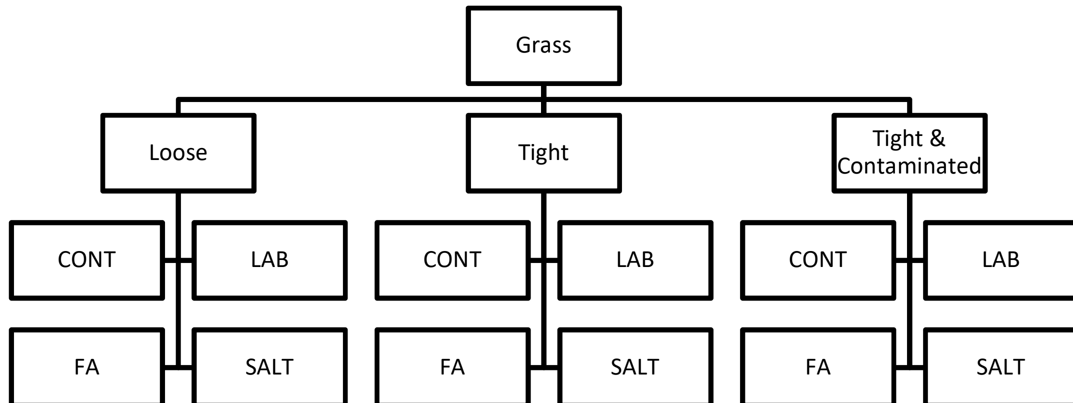

Figure 1. Schematic design of treatments.

The forage was mixed in laboratory and divided to 15 kg batches per silo. Additives were added to the forage in top of a 2.5 × 2.5 m plastic sheet by using a bottle with holes in the cap and forage was mixed well. All additives were added according to commercial dosages. Total amount of solution was always 250 ml for each 15 kg batches which is equal to 16,7 l/t FM. Formic acid solution contained 75 ml of formic acid-based product, LAB 15 g of bacteria and Salt 30 ml of salt based-product and rest of the solutions were water.

Forages were ensiled in cylindrical plastic silos with volume of 12 L. One batch of 15 kg was divided in two silos (loose and tight). Soil contamination treatment was done separately. All silos were filled to the same level and weight of FM were approximately 4.8 kg in loosely compacted silos and 6.6 kg in tightly compacted silos.

All the treatments had three replicates, so total number of silos was 12×3=36. Silos were capped with a plastic cover, a weight and a water bag. Silos were stored at room temperature and protected from light for 93 days before opening. Height of silage pile was measured before opening for density calculations. Silos were weighted before opening for calculation of DM ensiling losses. Top layer of the silos and visually spoiled silage were discarded, and rest of the silage were softly mixed for the final samples of every replicate.

Three raw material samples were taken before additive treatment for different analysis. First sample was frozen to -80 °C to avoid DNA breakdown. Later it was freeze-dried for DNA extraction. Second sample was analysed for DM, ash, CP, NDF, WSC, pepsin-cellulase solubility and buffering capacity. Third sample was used for analysis of aerobic bacteria, yeasts, moulds, Clostridia and mycotoxins Zearalenone and Deoxynivalenol. Botanical sample was taken using 0.5×0.5 m frame and cutting all the grass inside the frame at height of 5 cm. Botanical composition was analysed in laboratory by divining plant species in fractions and weighing those separately.

Three samples were taken from each silo after ensiling similarly than from raw plant material, described above. Silage DM, ash, CP, pH, ammonia N, ethanol, VFAs, formic acid for FA treated samples and lactic acid were analysed in Luke’s laboratory. One sample was used for DNA extraction and sequencing. DNA extraction was performed in Luke’s laboratory, but DNA sequencing was done in Finnish Functional Genomics Centre (Turku Bioscience, Turku, Finland). Third sample was for analysis of aerobic bacteria, yeast, moulds, *Clostridia* and mycotoxins in Luke’s laboratory.

5.2 Laboratory analyses

Dry matter of silages and raw materials was determined by drying samples at 105 °C for 16 hours and corrected for volatile compounds. Crude protein and ash were determined according to AOAC (1990): CP by method 968.06 (correction factor 6.25×N, using Leco FP 428 nitrogen analyzer (Leco, St. Joseph Michigan, USA)) and ash by method 942.05. Water soluble carbohydrates were determined according to Somogyi (1945), VFAs according to Huhtanen et al. (1998), lactic acid according to Haacker et al. (1983) and ammonia according to McCullough (1967). In vitro organic matter digestibility was determined according to Nousiainen et al. (2003) with correction equation of pepsin-cellulase solubility to in vivo digestibility by using data from Finnish in vivo digestibility trials (Huhtanen et al. 2006). NDF was determined by using ANKOM 220 Fiber Analyzer (ANKOM Technology, Macedon NY, USA) according to Van Soest et al. (1991) and with sodiumsulphite and expressed without ash.

Ethanol was detected with commercial kit (Cat. No. 10 176 290 035, Boehringer Mannheim GmbH, Mannheim, Germany) by using spectrophotometer according to given directions.

Yeasts and moulds were determined by cultivation on petri dish on Dichloran Rose Begal Chloramphenicol Agar medium (Lab M Ltd. Lab217, Lancashire, UK) plate with 50 µg/ml of oxytetracycline hydrochloride (AppliChem BioChemica A5257). The dishes were incubated at temperature of 25 °C and visually counted after days three and five. Aerobic bacteria dishes (Plate Count Agar, Lab M Ltd. Lab010, Lancashire, UK) were incubated at 30 °C for 72 hours.

Aerobic stability was measured in Luke's facilities using its standard method. Silages were put inside of polystyrene boxes and thermocouple wires were connected to data logger. Temperature was automatically measured after every ten minutes for 480 hours period. Silage sample was aerobic stable until its temperature increased 2 above ambient temperature. Ambient temperature was around 22 °C and it was automatically measured with similar data logger. All the samples were weighted before and after aerobic stability trial for estimation of aerobic losses.

Ensiling losses were estimated according to Knicky and Spörndly (2015) by using weight loss of sample during ensiling as an estimate of CO_2 production. For each mole of produced CO_2 during ensiling, 1 mole of H_2O is also produced. Thus, each gram of weight decrease because of CO_2 losses means that 0.44 g of DM was also lost as water. It's counted as an ensiling loss even if water is still in a silo. Total DM loss (g/kg initial DM) was then estimated to be decrease in weight of the silo multiplied by 1.44.

5.3 DNA extraction and sequencing

The DNA extraction was done by following the Repeated Bead Beating Plus Column (RBB+C) Method. Approximately 0.25 g of freeze dried and ground silage sample was added to tube with lysis buffer liquid (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate (SDS)) and 0.4 g of sterile zirconia beads. Homogenization was achieved with Mini-

Beadbeater™ (BioSpec Products, Bartlesville, OK, USA) in 3 minutes. Then the sample was incubated at 70 °C for 15 minutes and gently shook occasionally. Cell lysis was completed with centrifuge at 4 °C for 5 minutes at 16000 × g. The supernatant was moved to fresh tube and 300 µL of fresh lysis buffer was added. Homogenization, incubation and centrifugation were repeated one more time.

Precipitation of nucleic acids started by adding 260 µL of 10 M ammonium acetate to the tube, mixing it well and incubating in ice for 5 minutes. Then tube was centrifuged at 4 °C for 10 minutes at 16000 × g. The supernatant was transferred to two new tubes and on volume of isopropanol was added, mixed well and incubated on ice for 30 minutes. Then tubes were centrifuged again at 4 °C for 15 minutes at 16000 x g and removed with aspiration. Nucleic acid pellets were washed with 70 % ethanol and dried under vacuum. Then pellets were dissolved in 100µL of TE (Tris-EDTA) buffer and two aliquots were pooled again.

The RNA and protein removal and purification started by adding of 2 µL of DNase-free RNase (10 mg/mL) and incubating at 37 °C for 15 minutes. The 15 mL of proteinase K and 200 µL of Buffer AL (from QIAamp DNA Stool Mini Kit) were added and mixed well before incubation at 70 °C for 10 minutes. Then 200 µL of ethanol were added and mixed before moving it to QIAamp and centrifuged at 16000 × g for 1 minute. Then two buffers were added one by one (500 µL of both Buffer AW1 and AW2 (Qiagen) and centrifuged between those and after. Extra flow of supernatant through the column was discarded. The column was dried by centrifuging it at room temperature for 1 minute before 200 µL of Buffer AE (Qiagen) was added and incubated for 2 minutes. DNA was eluted with centrifuging it for 1 minute. Then DNA was divided into four new tubes and 2 µL of it was run on a 0.8 % gel to test the quality of extracted DNA.

DNA sequencing was based on 16S rRNA amplicons. Polymerase chain reaction (PCR) was used to produce more DNA. Primers for RNA gene region V4 were used in paired-ended 2 × 250 bp sequencing on Illumina MiSeq application (Illumina Inc., San Diego, Ca, USA). This method is good for small genome and targeted sequencing.

5.4 Calculation of the results

For final data only 16 bacteria with highest abundance in silages were selected. Totally 562 bacterial community were identified from samples. Those 16 bacteria were selected so that if abundance of bacteria were lower than 1% in at least 50 % of samples it was excluded from the data. Two dominant identified group were discarded from final data because those DNA strains do not originate from bacteria. They were order *Streptophyta* and family *Mitochondria* and they were dominant especially in raw material (*Streptophyta* 63.7% and *Mitochondria* 30.2% in relative abundance of 18 most dominant identified DNA, respectively).

The goal for bacteria identification was to go on genus-level but five of those 16 bacteria were only identified in family level. For other 11 bacteria genus-level was used in final dataset.

Fermentation coefficient (FC) was calculated based on DM, WSC and buffering capacity (BC) of raw material using following formula: FC = DM + (8 x WSC/BC) (Pahlow at al. 2002). Dry matter was used as % and WSC as % in DM of raw material.

5.5 Statistical analysis

Experimental data were analysed using a MIXED procedure (SAS Inc. 2002-2012, Release 9.4; SAS Institute Inc., Cary, NC, USA) of SAS with additive, compaction and soil contamination as fixed effects and replicates as a random effect. Least squares means and standard error of the means were reported per treatment and differences among treatment means were declared significant at 5% of probability. A pairwise comparison among treatment means was performed using a Tukey´s test. Bacterial composition was filtered using R-Studio software and taxa with overall average abundance of less than 1% in at least 50% of the data were excluded from the data set. The bacterial communities were converted into relative percent abundance and treatment effects on individual bacterial taxa were evaluated through the MIXED procedure above described. In order to explore the magnitude of associations within bacterial communities and between bacterial communities and

fermentation quality parameters, the variables were ordered based on a hierarchical cluster analysis of a Pearson correlation plot and a heat map originated from two-dimensional display was created to characterize the effects of bacteria species on fermentation quality.

6 Results

6.1 Raw material composition and fermentation quality of grass silages

Botanical composition of forage was timothy 81% and meadow fescue 19% in FM. Forage composition and microbial quality are listed in Table 5. Two compaction levels used in experiment were 424 kg/m^3 (146.7 kg DM/m^3) in loose silages and 583 kg/m^3 (201.7 kg DM/m^3) in tight silages.

Table 5. Composition and microbial quality of timothy grass.

Dry matter (DM), g/kg	346
Buffering capacity, g lactic acid/100g DM	6.2
Metabolizable energy, MJ/kg DM	11.7
in vitro organic matter digestibility, g/kg organic matter	796
Fermentation efficient	52.3
In DM, g/kg	
Ash	79
Crude protein	156
D-value	733
Neutral detergent fibre	503
Water soluble carbohydrates	137
Microbial quality	
Yeasts, cfu/g	5.4×10^5
Moulds, cfu/g	3.6×10^5
Total bacteria, cfu/g	5.72×10^7
Clostridia, spore/g	<3

cfu: colony-forming unit, estimates the number of viable bacteria or fungal cells in a sample. D-value: content of digestible organic matter in the DM.

Different treatments produced different fermentation profiles in final silages (Table 6). Use of additives decreased the pH in non-contaminated silages compared to CONT silages, except for SALT silage with tight compaction in which was similar to CONT with tight compaction. In soil contaminated silages

FA had higher pH than other silages. On average, loose compaction silages resulted in higher silage pH than tight compaction and non-contaminated silages had higher pH than contaminated silages ($P<0.01$). Compaction and contamination treatments did not affect pH of FA silages. LAB treated silages had lower pH compared to all other additive treatments in all categories. Soil contamination significantly decreased the pH of CONT and SALT silages compared to non-contaminated CONT and SALT silages.

Lactic acid production was higher in LAB silages compared to others. On average, of all treatments both tight compaction and soil contamination promoted it ($P<0.01$) but there were no differences in lactic acid production within compaction or contamination in FA and LAB treated silages. On average, soil contamination increased the amount of acetic acid in silages ($P<0.01$). That happened because CONT and SALT silages with soil contamination had considerably higher amount of acetic acid than any other silage. The total concentration of fermentation acids was high in LAB silages compared to other additive treatments, followed by CONT and SALT and then FA. Compaction did not affect the average level of ammonia N in silages. Use of silage additives decreased the concentration of ammonia N in silages compared to CONT silages except for SALT with soil contamination which was similar than CONT with soil contamination. Production of butyric and propionic acids were relatively low in all samples.

Residual WSC in silage varied between 5 and 195 g/kg DM (in CONT, tight, soil contaminated and in FA, tight, non-contaminated, respectively) and on averages were lower in soil contaminated silages ($P<0.01$) compared to non-contaminated silages. However, there were no differences in residual WSC in LAB and FA with different compaction or contamination treatments. In all categories FA preserved WSC better than other treatments.

Compaction did not affect aerobic stability, but soil contamination improved it on average ($P<0.01$) because aerobic stability times of tight and soil contaminated CONT and SALT silages were relatively long (Table 6). SALT with tight compaction and soil contamination even reached the maximum 480 h aerobic stability. Non-contaminated CONT and SALT silages had some DM ensiling

losses. With contamination other than FA treatment resulted in moderate DM losses.

Only bacteria were identified with DNA techniques, whereas other microbes were studied only with plate cultivation methods. No significant differences were found in yeasts between compaction or contamination treatments. CONT silages had numerically higher amount of yeast than additive treated silages. Soil contaminated silages had less moulds than non-contaminated silages ($P<0.01$) and no differences between compactions were found. Overall numbers of yeasts and moulds were relatively low. CONT silages with soil contamination had higher number of mycotoxins than other silages (zearalenone 1598 ppb, deoxynivalenol 558 ppb) when only CONT and FA silages were analyzed. *Clostridia* was present only in small numbers, biggest abundances were with non-contaminated tight CONT (42 spores/g) and FA (34 spores/g).

Table 6. Fermentation quality, chemical composition, aerobic stability, ensiling losses and microbial composition of grass silage with different additives under two compaction (Comp.) and soil contamination (Soil.) levels.

Contamination	Non-contaminated								Soil contaminated				SEM[1]	P-value[2]	
Compaction	Loose				Tight				Tight						
Additive	CONT	FA	LAB	SALT	CONT	FA	LAB	SALT	CONT	FA	LAB	SALT		Comp	Soil
Dry matter (DM), g/kg	331[b]	345[ab]	348[ab]	337[ab]	332[ab]	345[ab]	344[ab]	335[ab]	341[ab]	348[a]	339[ab]	341[ab]	3.2	0.67	0.20
pH	5.78[a]	4.85[c]	4.02[ef]	5.48[b]	5.53[b]	4.78[c]	4.00[f]	5.36[b]	4.26[d]	4.83[c]	4.01[f]	4.21[de]	0.039	<0.01	<0.01
Ammonia N, g/kg N	64[a]	26[c]	21[c]	42[b]	59[a]	25[c]	21[c]	43[b]	43[b]	25[c]	22[c]	43[b]	2.2	0.46	0.03
Chemical composition, g/kg DM															
Ash	88[a]	82[dc]	85[abcd]	88[a]	86[ab]	82[d]	85[abcd]	86[ab]	86[ab]	83[bcd]	84[bcd]	86[abc]	0.8	0.11	0.80
Crude protein	177[a]	164[c]	172[abc]	177[a]	172[abc]	166[bc]	171[abc]	175[ab]	170[abc]	169[abc]	170[abc]	171[abc]	1.9	0.27	0.35
Sugars	87[cd]	187[a]	73[d]	120[bc]	120[bc]	195[a]	76[d]	135[b]	5[e]	181[a]	66[d]	6[e]	8.2	0.02	<0.01
Ethanol	29.9[ab]	7.8[de]	3.6[e]	31.8[a]	16.5[c]	4.7[e]	3.2[e]	22.0[bc]	16.4[c]	2.5[e]	3.9[e]	15.0[cd]	1.56	<0.01	0.06
Acids, g/kg DM															
Formic[3]	0[d]	0.9[c]	0[d]	0[d]	0[d]	1.7[b]	0[d]	0[d]	0[d]	2.9[a]	0[d]	0[d]	0.14	0.07	0.01
Lactic (LA)	12.7[d]	1.2[e]	113.1[a]	12.4[d]	21.8[c]	1.6[e]	114.5[a]	17.7[c]	86.2[b]	0.7[e]	115.5[a]	87.8[b]	0.93	<0.01	<0.01
Acetic	7.5[c]	7.5[c]	12.8[b]	9.0[bc]	8.3[bc]	7.7[c]	12[bc]	9.9[bc]	30.4[a]	7.4[c]	9.4[bc]	25.9[a]	0.95	0.72	<0.01
Propionic[3]	0.15	0	0.08	0.11	0.17	0	0.10	0.09	0.24	0.34	0.11	0.23	0.100	0.94	0.06
Butyric	0.91	0.28	0.03	0.19	0.29	0.37	0.05	0.16	0.03	0.85	0.03	0.03	0.288	0.51	0.94
Total volatile fatty acids	8.64[bc]	7.82[c]	12.90[b]	9.39[bc]	8.76[bc]	8.12[bc]	12.12[bc]	10.19[bc]	30.76[a]	8.82[bc]	9.55[bc]	26.18[a]	0.964	0.87	<0.01
Total fermentation acids	21.3[d]	9.0[e]	126.0[a]	21.8[d]	30.5[c]	9.7[e]	126.6[a]	27.9[c]	116.9[b]	9.5[e]	125.0[a]	114.0[b]	1.16	<0.01	<0.01
LA/total fermentation acids	0.59[d]	0.12[e]	0.90[a]	0.57[d]	0.71[bc]	0.15[e]	0.91[a]	0.63[cd]	0.74[bc]	0.07[e]	0.92[a]	0.77[b]	0.022	<0.01	0.12
Total fermentation products	51[b]	17[c]	130[a]	54[b]	47[b]	14[c]	130[a]	50[b]	133[a]	12[c]	129[a]	129[a]	1.7	0.05	<0.01
Aerobic stability, h[4]	41[d]	109[bcd]	118[bc]	46[cd]	73[bcd]	98[bcd]	133[b]	48[cd]	469[a]	127[b]	90[bcd]	480[a*]	14.8	0.37	<0.01
Ensiling losses, g/kg of initial DM	89[a]	13[fg]	3[i]	60[d]	79[b]	10[gh]	17[f]	68[c]	44[e]	4[hi]	41[e]	43[e]	1.2	0.01	<0.01
Yeasts, cfu/g	4.7×10^{5}	2.9×10^{3}	1.6×10^{3}	1.4×10^{4}	1.4×10^{4}	4.3×10^{2}	3.0×10^{2}	1.3×10^{3}	1.0×10^{2}	9.6×10^{2}	4.0×10^{4}	1.0×10^{2}	9.4×10^{4}	0.09	0.93
Moulds, cfu/g	3.1×10^{3}[b]	2.2×10^{3}[b]	3.2×10^{2}[b]	1.4×10^{4}[a]	5.2×10^{3}[b]	4.1×10^{2}[b]	3.1×10^{2}[b]	1.4×10^{4}[a]	1.0×10^{2}[b]	3.1×10^{3}[b]	4.6×10^{2}[b]	3.0×10^{2}[b]	1.6×10^{3}	0.94	<0.01
Clostridia, spore/g	-	-	-	-	42	34	3	7	3	13	3	14	16.3	-	0.28
Zearalenone, ppb	403	371	-	-	234	221	-	-	1598	313	-	-	-	-	-
Deoxynivalenol, ppb	299	297	-	-	322	385	-	-	558	252	-	-	-	-	-

CONT: Control, FA: Formic acid based additive, LAB: Lactic acid bacteria additive, SALT: Salt based additive.
Values with same letter in a row are not significantly different at 5% Tukey test. If there are no differences in Tukey test, letters are removed.
[1]Standard error of the mean. [2]Effect of compactions and soil contamination. [3]Corrected for its amount in the FA based additive. [4]Time taken to increase the temperature of samples by 2 °C above the ambient temperature (22 °C). [*]Treatment did not reach the threshold during the evaluation period.

6.2 Bacterial communities in grass silages

Bacterial composition of raw material was completely different than in any final silage, because selected 16 bacteria were totally only 5.8 % of raw material sequenced bacteria (Figure 2). In silages the real abundances of those 16 most predominant bacteria were 50.2 - 95.7 % of all identified bacteria (Figure 2). *Lactobacillaceae* family and *Lactobacillus* genus were minor (abundance <1%) in raw material but they became dominant in the silages. FA-treated silages were more diverse and real abundance of these 16 bacteria were just little bit over 50% in FA silages.

Ensiling process made a clear compositional shift in microbial populations. *Shingomonas* and *Stenotrophomonas* were dominant bacteria in raw material whereas family *Lactobacillus* and genus *Lactobacillaceae* were low in relative abundance but after ensiling they were among the most dominant bacteria in all samples with a share up to 60 % in LAB treated silages. Other dominant genera after fermentation were *Sphingomonas*, *Pediococcus* and *Stenotrophomonas*. Formic acid led to different microbial population than other treatments, relative abundance of *Streptomonas*, *Mycoplana* and *Pedobacter* genera were higher in FA treated silages compared to other treatments. In turn *Lactobacillus* and *Lactobacillaceae* were not so dominant in FA silages than they were in other silages. In general FA and SALT led to more diverse microbiome in silage than CONT and LAB.

SALT and CONT silages were higher in abundance of family *Leuconostocaceae* than other silages. In loose and non-contaminated silages abundances of *Leuconostocaceae* were as follows: CONT 2.08, FA 0.58, LAB 0.00 and SALT 1.98.

Soil contamination reduced the abundance of other family *Lactobacillaceae* bacteria but boosted the growth of genus *Lactobacillus* bacteria, especially in Control and Salt silages. Abundances of genera *Cryocola*, *Mycoplana*, *Devosia*, *Rhizobium*, *Shingomonas* and *Stenotrophomonas* were lower in soil contaminated treatments in CONT, LAB and SALT, because *Lactobacillus* was so dominant in those silages. Two biggest abundances were genus *Lactobacillus* in CONT (83.12) and SALT (83.30) silages under soil

contamination. Formic acid led to much more diverse population even with soil contamination. A few significant differences between compactions were observed such as *Pediococcus* and *Lactobacillus*, which were more common in tight and *Agrobacterium* which was more common in loose compaction. Soil contamination affected fermentation profile more than compaction in this experiment.

When comparing modulation of bacterial communities between ensiling managements factors, relative abundances of bacteria remained close to each other between compaction and contamination in LAB and FA silages. Only small differences were observed such as in FA silages abundance of *Devosia* was higher in tight non-contaminated silage compared to loose and tight with soil contamination. In CONT and SALT silages variation between treatments was larger. Especially relative abundances on *Lactobacillus* and *Pediococcus* changed a lot between compaction and contamination in CONT and SALT silages. Also, real abundances of 16 selected bacteria in CONT and SALT silages variated a lot between treatments, CONT between 59.9-94.9 % and SALT between 68.5-95.7 %. In LAB and FA silages real abundances were much more stable between treatments.

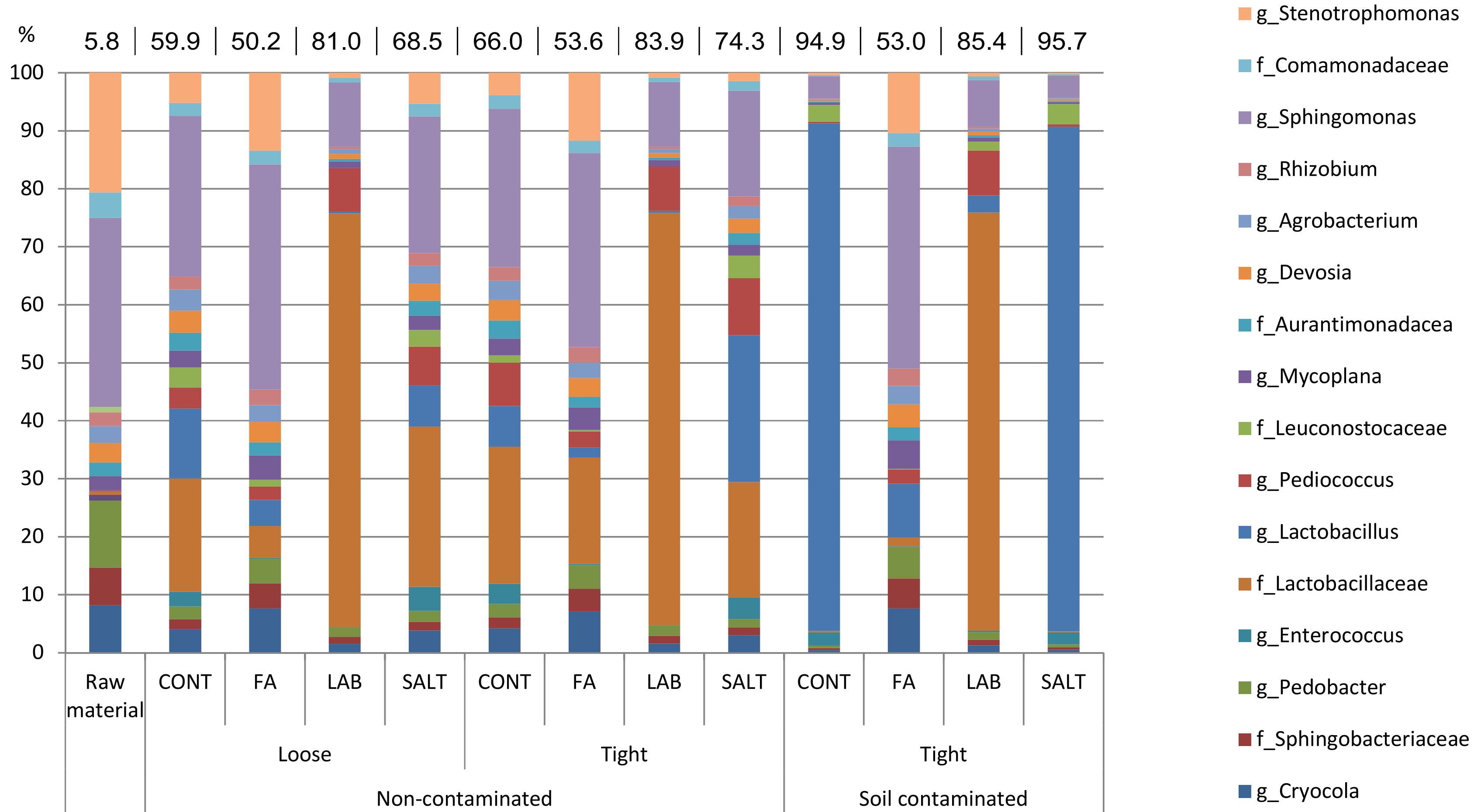

Figure 2. Abundances of silage bacterial populations of grass silage treated with different additives under two compaction and soil contamination levels. Bars show relative (scaled to 100 %) abundances and numeric value on top of the bar show real abundance of group of selected 16 bacteria in total identified bacteria (%). Numeric values presented in Appendix 1.

Some patterns are seen in correlations between bacterial communities and fermentation quality parameters (Figure 3). Genera *Enterococcus*, *Lactobacillus* and *Pediococcus* and families *Lactobacillaceae* and *Leuconostocaceae* represented different group of bacteria within these 16 bacteria. Those five bacteria are part of phylum *Firmicute* and class *Bacilli. Firmicute* is a main group of gram-positive bacteria including all *Bacilli* and *Clostridia* bacteria. Those bacteria belonging to *Firmicutes* seemed to improve production of fermentation products and acids even though not all the correlations are significant. Overall, those five bacteria can be classified as lactic acid bacteria. All the other 11 bacteria are not part of phylum *Firmicutes* and in heat map they inhibited production of total fermentation products and acids, total VFA, and ratio of lactic acid by total fermentation products (P<0.05).

Most of the bacteria correlated positively with pH, for example genus *Aurantimonadaceae* had correlation of 0.95 (P<0.01) and genus *Agrobacterium* 0.91 (P<0.01). Only family *Lactobacillaeae* (-0.49, P<0.01) was clearly and significantly correlated with decrease of pH, with genus *Lactobacillus* (-0.28, P=0.10) result was not significant. Family *Lactobacillaeae* was also the only one with clear positive correlation with lactic acid production (0.69, P<0.01). All bacteria which are not *Firmicutes* had clear and significant negative correlation with lactic acid and positive correlation to residual WSC.

Lactobacillus correlated strongly and positively with aerobic stability (r=0.93, P<0.01) whereas non-*Firmicutes* such as family *Comamonadaceae* (r=-0.78, P<0.01), genus *Agrobacterium* (r=-0.69, P<0.01), genus *Devosia* (r=-0.70, P<0.01) and genus *Shingomonas* (r=-0.69, P<0.01) correlated negatively with it. Lactobacillus had positive correlation with acetic acid (r=0.93, P<0.01) and so did family *Leuconostocaceae* (r=0.46, P=0.05).

Some correlations to ethanol were found. Enterococcus (r=0.80, P<0.01) and family *Leuconostocaceae* (r=0.54, P<0.01) were Firmicutes with positive correlation to ethanol. Family *Aurantimonadaceae* (r=0.57, P<0.01), Devosia (r=0.38, P<0.05) and *Agrobacterium* (r=0.48, P<0.01) were non-Firmicutes with significant positive correlation to it. Bacteria with negative and significant correlation to ethanol were family Sphingobacteriaceae (r=-0.40, P<0.05), Pedobacter (r=-0.42, P<0.05) and family *Lactobacillaeae* (r=-0.37, P<0.05).

	g__Cryocola	f__Sphingobacteriaceae	g__Pedobacter	g__Enterococcus	f__Lactobacillaceae	g__Lactobacillus	g__Pediococcus	f__Leuconostocaceae	g__Mycoplana	f__Aurantimonadaceae	g__Devosia	g__Agrobacterium	g__Rhizobium	g__Sphingomonas	f__Comamonadaceae	g__Stenotrophomonas
DM	0.1051	0.3669	0.4351	-0.5140	0.0730	-0.0510	-0.1250	-0.4220	0.1245	-0.3990	-0.2100	-0.2620	-0.0740	0.0197	-0.1440	0.2323
	0.5418	0.0277	0.0080	0.0013	0.6723	0.7699	0.4666	0.0103	0.4695	0.0160	0.2188	0.1228	0.6663	0.9092	0.4029	0.1728
pH	0.5650	0.2008	0.2121	0.5208	-0.4940	-0.2790	-0.0430	0.1690	0.6199	0.9487	0.8900	0.9141	0.8100	0.6927	0.8162	0.4083
	0.0003	0.2404	0.2142	0.0011	0.0022	0.0992	0.8041	0.3244	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0134
Ammonia	-0.0640	-0.3850	-0.3600	0.7244	-0.4590	0.2808	-0.1590	0.5231	0.0196	0.5990	0.3969	0.4719	0.2772	0.0894	0.2841	-0.1100
	0.7126	0.0204	0.0308	<.0001	0.0048	0.0971	0.3551	0.0011	0.9097	0.0001	0.0165	0.0037	0.1017	0.6043	0.0931	0.5217
Ash	-0.3790	-0.6160	-0.4960	0.7331	-0.0460	0.2357	0.1882	0.3994	-0.2420	0.3349	0.1330	0.2591	-0.0020	-0.1760	0.1383	-0.3660
	0.0226	<.0001	0.0021	<.0001	0.7909	0.1664	0.2717	0.0158	0.1553	0.0459	0.4395	0.1271	0.9928	0.3036	0.4211	0.0283
Crude protein	-0.2830	-0.4130	-0.2620	0.5494	0.1234	0.0028	0.3578	0.1828	-0.1110	0.3210	0.1854	0.3065	0.0646	-0.0520	0.2442	-0.2760
	0.0944	0.0123	0.1231	0.0005	0.4733	0.9871	0.0321	0.2860	0.5212	0.0563	0.2790	0.0691	0.7083	0.7645	0.1511	0.1038
Sugar	0.9445	0.8487	0.8430	-0.2870	-0.2270	-0.6820	-0.0030	-0.4610	0.9032	0.5436	0.7425	0.6581	0.8318	0.8816	0.7631	0.8049
	<.0001	<.0001	<.0001	0.0895	0.1836	<.0001	0.9883	0.0047	<.0001	0.0006	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Ethanol	-0.0690	-0.4040	-0.4160	0.7999	-0.3680	0.1961	-0.0330	0.5390	-0.0005	0.5741	0.3782	0.4795	0.2658	0.0915	0.3280	-0.0650
	0.6903	0.0146	0.0117	<.0001	0.0274	0.2517	0.8498	0.0007	0.9976	0.0003	0.0230	0.0031	0.1171	0.5957	0.0509	0.7044
Formic	0.6815	0.8655	0.7845	-0.4650	-0.3610	-0.2480	-0.4090	-0.4070	0.6558	0.0922	0.3356	0.2156	0.4530	0.5603	0.2694	0.6543
	<.0001	<.0001	<.0001	0.0043	0.0304	0.1454	0.0134	0.0138	<.0001	0.5927	0.0454	0.2066	0.0055	0.0004	0.1121	<.0001
Lactic	-0.8540	-0.6070	-0.5450	-0.2130	0.6855	0.2976	0.3104	0.0363	-0.8440	-0.7930	-0.8770	-0.8340	-0.9130	-0.8550	-0.7930	-0.7590
	<.0001	<.0001	0.0006	0.2118	<.0001	0.0779	0.0654	0.8336	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Acet	-0.7230	-0.5960	-0.6650	0.3033	-0.2190	0.9315	-0.3670	0.4565	-0.7400	-0.6100	-0.7160	-0.6900	-0.6930	-0.7700	-0.7690	-0.5290
	<.0001	0.0001	<.0001	0.0721	0.1986	<.0001	0.0275	0.0051	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0009
Prop	-0.1610	0.0978	0.0976	0.1139	-0.1890	0.2639	-0.2540	0.1582	-0.0550	0.0095	-0.0510	-0.0660	-0.0360	-0.0180	-0.0820	-0.1490
	0.3485	0.5705	0.5713	0.5083	0.2702	0.1199	0.1349	0.3568	0.7497	0.9564	0.7690	0.7020	0.8329	0.9174	0.6339	0.3853
Valeric	0.5178	0.4079	0.3009	0.1121	-0.6540	0.1232	-0.3980	0.0780	0.4710	0.2102	0.3574	0.3109	0.4500	0.3640	0.2555	0.5032
	0.0012	0.0135	0.0746	0.5149	<.0001	0.4740	0.0162	0.6513	0.0037	0.2186	0.0324	0.0649	0.0059	0.0291	0.1326	0.0018
But	0.3625	0.2238	0.2611	-0.1020	-0.2430	-0.2010	-0.2120	0.1152	0.3879	0.3444	0.3981	0.3783	0.3795	0.3385	0.2486	0.2562
	0.0298	0.1895	0.1240	0.5530	0.1535	0.2393	0.2147	0.5034	0.0194	0.0397	0.0162	0.0229	0.0224	0.0434	0.1438	0.1315
Total VFA	-0.7080	-0.5830	-0.6510	0.3031	-0.2470	0.9364	-0.3950	0.4733	-0.7210	-0.5930	-0.6970	-0.6720	-0.6740	-0.7540	-0.7630	-0.5190
	<.0001	0.0002	<.0001	0.0724	0.1463	<.0001	0.0171	0.0035	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0012
Total Ferm Ac	-0.8890	-0.6430	-0.5960	-0.1530	0.5968	0.4085	0.2298	0.1012	-0.8820	-0.8160	-0.9090	-0.8660	-0.9380	-0.8970	-0.8410	-0.7740
	<.0001	<.0001	0.0001	0.3722	0.0001	0.0134	0.1776	0.5571	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
LA by total Ferm Ac	-0.8630	-0.8320	-0.7190	0.2332	0.6313	0.2403	0.5208	0.2487	-0.7920	-0.3630	-0.5770	-0.4770	-0.6860	-0.7420	-0.4890	-0.8630
	<.0001	<.0001	<.0001	0.1710	<.0001	0.1580	0.0011	0.1435	<.0001	0.0296	0.0002	0.0033	<.0001	<.0001	0.0025	<.0001
Total Ferm Prod	-0.9480	-0.7610	-0.7140	0.0079	0.5494	0.4705	0.2345	0.2201	-0.9260	-0.7370	-0.8750	-0.8080	-0.9290	-0.9230	-0.8140	-0.8270
	<.0001	<.0001	<.0001	0.9634	0.0005	0.0038	0.1686	0.1970	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Aerobic Stability	-0.6110	-0.4430	-0.5330	0.1756	-0.3220	0.9304	-0.5220	0.4120	-0.6420	-0.6270	-0.6950	-0.6940	-0.6350	-0.6900	-0.7810	-0.3910
	<.0001	0.0069	0.0008	0.3056	0.0552	<.0001	0.0011	0.0125	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0184
Ensiling Losses	-0.1680	-0.4840	-0.4580	0.7712	-0.2070	0.1575	0.1364	0.5272	-0.0920	0.5749	0.3386	0.4293	0.2006	0.0144	0.2974	-0.2610
	0.3274	0.0028	0.0050	<.0001	0.2254	0.3589	0.4276	0.0010	0.5937	0.0002	0.0434	0.0090	0.2409	0.9335	0.0782	0.1240

Figure 3. Pearson correlations between bacterial communities and fermentation quality of timothy grass silage. *Firmicutes* separated with black lines in the middle of the figure. Colours represent correlations as explained below.

Correlations between bacterial communities showed that some of them boosted the growth of each other and some inhibited the abundance of competitor bacteria (Figure 4). Strong and positive correlation was found e.g. between these three genera: *Cryocola* and *Pedobacter*, *Cryocola* and *Sphingobacteriaceae* and *Sphingobacteriaceae* and *Pedobacter*. The strongest interaction between two genera was a positive correlation between *Agrobacterium* and *Devosia* ($r=0.98$, $P<0.01$).

Firmicutes correlated mostly negatively with each other and to all the other bacteria. Five bacteria of *Firmicutes* are a yellow-orange-red stripe between green non-*Firmicutes* in Figure 4. Some positive correlations were found within *Firmicutes*: *Enterococcus* to *Lactobacillus*, family *Lactobacillaceae* to *Pediococcus*, and family *Leuconostocaceae* to *Enterococcus* and to *Lactobacillus*. Genus *Lactobacillus* had remarkable clear negative correlation with all the non-*Firmicutes*.

Non-*Firmicutes* had positive and significant correlation within each other except families *Aurantimonadaceae* and *Sphingobacteriaceae* (Figure 4). Family *Aurantimonadaceae* was the only one with positive and significant correlation to any of the *Firmicutes*; correlation to genus *Enterococcus*.

	g_Cryocola	f_Sphingobacteriaceae	g_Pedobacter	g_Enterococcus	f_Lactobacillaceae	g_Lactobacillus	g_Pediococcus	f_Leuconostocaceae	g_Mycoplana	f_Aurantimonadaceae	g_Devosia	g_Agrobacterium	g_Rhizobium	g_Sphingomonas	f_Comamonadaceae	g_Stenotrophomonas
g_Cryocola	1															
f_Sphingobacteriaceae	0.875	1														
	<.0001															
g_Pedobacter	0.848	0.951	1													
	<.0001	<.0001														
g_Enterococcus	-0.231	-0.517	-0.528	1												
	0.1749	0.0013	0.0009													
f__Lactobacillaceae	-0.395	-0.258	-0.134	-0.409	1											
	0.0171	0.1287	0.4350	0.0132												
g_Lactobacillus	-0.607	-0.551	-0.651	0.454	-0.453	1										
	<.0001	0.0005	<.0001	0.0055	0.0055											
g_Pediococcus	-0.186	-0.249	-0.13	0.035	0.739	-0.492	1									
	0.2763	0.1437	0.4494	0.8385	<.0001	0.0023										
f_Leuconostocaceae	-0.419	-0.546	-0.611	0.546	-0.310	0.576	-0.108	1								
	0.0109	0.0006	<.0001	0.0006	0.0659	0.0002	0.5321									
g_Mycoplana	0.963	0.846	0.854	-0.186	-0.364	-0.633	-0.131	-0.445	1							
	<.0001	<.0001	<.0001	0.2773	0.0289	<.0001	0.4462	0.0066								
f_Aurantimonadaceae	0.639	0.326	0.374	0.397	-0.369	-0.450	0.052	0.001	0.714	1						
	<.0001	0.0527	0.0248	0.0165	0.0268	0.0059	0.7656	0.994	<.0001							
g_Devosia	0.825	0.557	0.606	0.172	-0.386	-0.565	-0.016	-0.188	0.884	0.941	1					
	<.0001	0.0004	<.0001	0.3157	0.0201	0.0003	0.9257	0.2731	<.0001	<.0001						
g_Agrobacterium	0.749	0.444	0.508	0.265	-0.34	-0.553	0.057	-0.139	0.822	0.958	0.977	1				
	<.0001	0.0066	0.0016	0.1179	0.0425	0.0005	0.7415	0.4191	<.0001	<.0001	<.0001					
g_Rhizobium	0.889	0.648	0.681	0.127	-0.454	-0.535	-0.069	-0.207	0.924	0.875	0.959	0.934	1			
	<.0001	<.0001	<.0001	0.4602	0.0054	0.0008	0.6884	0.2266	<.0001	<.0001	<.0001	<.0001				
g_Sphingomonas	0.948	0.809	0.821	-0.120	-0.348	-0.663	-0.102	-0.39	0.969	0.794	0.917	0.866	0.936	1		
	<.0001	<.0001	<.0001	0.4867	0.0373	<.0001	0.5524	0.0186	<.0001	<.0001	<.0001	<.0001	<.0001			
f_Comamonadaceae	0.789	0.536	0.609	0.151	-0.219	-0.669	0.149	-0.282	0.856	0.909	0.952	0.960	0.932	0.900	1	
	<.0001	0.0007	<.0001	0.3795	0.1987	<.0001	0.3865	0.0952	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		
g_Stenotrophomonas	0.856	0.784	0.732	-0.282	-0.426	-0.425	-0.409	-0.417	0.829	0.434	0.643	0.581	0.719	0.779	0.619	1
	<.0001	<.0001	<.0001	0.0951	0.0097	0.0097	0.0132	0.0114	<.0001	0.0082	<.0001	0.0002	<.0001	<.0001	<.0001	

Figure 4. Pearson correlations and p-values between bacterial communities. Colours represent correlations as explained below.

Correlation		
	1	Positive correlation
	0	No correlation
	-1	Negative correlation

7 Discussion of the results

Forage had relatively high DM and WSC concentrations and D-value (733 g/kg DM). With mediate level of metabolizable energy this forage had typical composition for forage in Finland and Northern Europe according to Huhtanen et al. (2006).

One objective of this experiment was to have a wide range of differently fermented silages to be able to find correlations between fermentation and bacterial communities. Clear differences were observed for bacterial community profile, fermentation quality and chemical composition of grass silages treated with different additives and variable management factors. Composition of silages was affected by the additive so that LAB converted WSC to lactic acid whereas FA preserved WSC and had low concentration of lactic acid in silages. Both FA and LAB treated non-contaminated silages were in overall well-preserved compared to CONT and SALT silages, when judged by concentration of ethanol and ammonia N, amount of ensiling DM losses and pH.

Soil contamination led to a wild-type of fermentation in SALT and CONT silages and in this experiment extensive acetic and lactic acid fermentation improved some parameters such as aerobic stability of silages compared to non-contaminated ones. However, Pauly and Wyss (2018) concluded that without an appropriate air stress test untreated control silages (and maybe other challenging silages as well) can sometimes show better aerobic stability than additive treated silages. Completely air-tight silos are hard to achieve in farm scale. Additive treated silages might work relatively better with air stress than in air-tight laboratory scale silos compared to control silages. In this experiment soil contaminated SALT and CONT silages had higher amount of lactic acid, acetic acid and VFAs compared to non-contaminated SALT and CONT silages. Especially amount of acetic acid could be responsible for long aerobic stability of soil contaminated CONT and SALT because it has been proven to be effective to improve aerobic stability (Oude Elferink et al. 2001, Danner et al. 2003).

Tight compaction of forage decreased the average of pH and ethanol and it is strongly recommended with all kind of additives. Tight compaction decreased the final pH in CONT and ethanol in CONT and SALT silages compared to loose compaction. In practical situations compaction level can vary a lot and potentially compromise the fermentation quality of silages at least in some parts of the silo. Both compactions were still below the suggestion density of 210 kg DM/m^3 (Wilkinson and Davies 2012) because loose compaction had density of 147 kg DM/m^3 and tight compaction 202 kg DM/m^3.

Loosely compacted silage was connected to decreased silage quality with maize silage specially at feed-out phase (Brüning et al. 2018) even though delayed sealing of silo impacted silage quality more. They also mentioned that before sealing the silo, loose compaction (194 kg DM/m^3) caused higher silage temperature than tight compaction (234 kg DM/m^3).

Amounts of residual WSC were minor in soil contaminated CONT and SALT silages, which is related to extensive fermentation. No differences were found in residual WSC of FA and LAB silages between different management factors. Amount of WSC were always mediate in LAB and high in FA silages. Usually low amount of residual WSC signify that fermentation could have needed more substrates. In this case wild-type fermentation was wide-ranging due diverse microbe activity. Pure lactic acid fermentation alone decreases pH so quickly, that all the WSC are usually not consumed as fermentation substrates but this requires enough WSC in raw material. LAB treated silages had significantly lower level of residual WSC than FA treatments, but still levels were similar through compaction and contamination. This with almost identical level of LA between LAB treatments means that lactic acid fermentation was strong enough to transform some WSC to LA and to preserve some of it compared to wild type of fermentation of CONT and SALT silages with soil contamination. Extensive and wild lactic and acetic acid fermentation used majority of WSC to produce variety of different fermentation products.

Both CONT in non-contaminated silages had high pH despite relatively high amount of WSC in raw material and there were plenty of residual WSC in those silages. Amount of ammonia N in total N where higher in those silages compared to other silages, signalling protein degradation.

For some reason, natural epiphytic bacteria were not able to decrease pH efficiently whereas Control in soil contaminated silage went through an extensive fermentation. Probably composition of natural bacteria was not suitable for efficient lactic acid fermentation in non-contaminated CONT silages. Similarly, non-contaminated SALT silages resulted in high residual WSC concentration and higher pH and amount of ammonia N compared to FA and LAB silages.

Fermentability coefficient (FC) considers buffering capacity, DM and WSC concentrations of raw material and for this plant material it was 52,28. Generally FC above 45 indicates good fermentability (Pahlow at al. 2002) so this raw material should have been suitable for lactic acid fermentation.

Within all treatments LAB was able to decrease pH of silages to around 4.0 and FA to pH 4.8, when pH in CONT and SALT silages variated more. Amounts of lactic acid were higher in all LAB treated silages than it is recommended (Artturi®-system: Lactic and formic acid combined 35-80 g/kg DM). High amount of lactic acid is beneficial for ensiling the forage but might decrease the DM intake of silage (Huhtanen et al. 2002, Krizsan and Randby 2007). Huhtanen et al. (2002) mentioned that concentration of total acids decreased the DM intake clearly, but lactic acid:total acids- ratio became also significant, meaning that other acids produced by heterolactic fermentation decreased DM intake more than lactic acid by homolactic fermentation.

Formic acid treatment seemed to restrict the fermentation even with contaminated poor-quality grass. The pH of FA treated contaminated silage was higher than in other treatments but similar than in other FA silages. For this DM content (346 g/kg DM) the pH of FA silages were higher than it is recommended in silage quality evaluation systems such as Artturi® (target <4.4 in 321 g DM/kg). Formic acid seemed to have good ability to stabilize even hygienically poor silage and it has been proven to be effective in difficult conditions such as with very low DM content (<200 g/kg FM) (Kung et al. 2003, Lorenzo and O'Kiely 2008, Seppälä et al. 2016).

No statistical differences were found in amount of butyric or propionic acids and yeasts between treatments. There was higher amount of moulds in SALT non-contaminated silage compared to other non-contaminated silages.

Abundances of bacterial communities were different before and after fermentation as predicted in hypothesis. In raw material many aerobic epiphytic bacteria are dominant, but those are suppressed during fermentation process when facultative and strictly anaerobic bacteria start to dominate after aerobic phase of ensiling (Merry et al. 2000). With bacterial inoculants the added bacterium is often still not the most dominant one in silage. Sometimes it just modulates the shift of whole bacterial community during ensiling (Xu et al. 2019).

Relative abundances of FA and LAB were different from each other, but both remained almost the same even though compaction and contamination changed. Also, share of selected 16 bacteria from all bacteria was stable in FA and LAB. Effective silage additive seems to be able to control silage microbiome also in difficult ensiling conditions. In CONT and SALT silages variation was larger than in FA and LAB silages, mostly because contaminated CONT and SALT silages were clearly different from any other silage microbiome. CONT and SALT treatments were not able to control fermentation profile when poor hygiene conditions occured.

Correlation between fermentation parameters and bacterial abundance could mean that bacteria promote the amount of fermentation product or the other way. For example, strong positive correlation between formic acid and genera *Cryocola* and *Pedobacter* and family *Sphingobacteriaceae* more likely means that formic acid boosted the abundance of those bacteria, because formic acid is often found only in silages treated with formic acid based additive. Fermentation does not usually produce more of it (Sleat and Mah 1984, Krizsan and Randby 2007) even though heterolactic pathway can produce formate as part of fermentation (McDonald et al. 1991 p.91-92) An opposite example is a positive correlation between genus *Enterococcus* and ethanol, which probably indicate that bacteria promote the production of ethanol in silage. Ethanol could be produced by these bacteria itself or by some other microbe which has positive correlation with genus *Enterococcus*.

Enterococcus, *Lactobacillus* and *Pediococcus* species are commonly used as silage additives (Merry et al. 2000). Those with families *Leuconostocaceae* and *Lactobacillaceae* formed a distinct group of bacteria which somehow correlated

with fermentation parameters related to strong and favourable fermentation. Family *Lactobacillaceae* correlated significantly to lactic acid production and low pH. Residual WSC was also negatively correlated with *Lactobacillus* and family *Leuconostocaceae*. Even the not significant results within that group were clearly different than results with other 11 bacteria. *Enterococcus* and family *Leuconostocaceae* both had positive correlation to ammonia and ensiling losses which are not wanted results in ensiling. Still they were closer to desirable fermentation bacteria than to other 11 bacteria in heatmap correlations (Figure 3).

All the bacteria in group of five desirable fermentation bacteria belong to same order *Lactobacillales*, part of phylum *Firmicutes.* They were the only *Firmicutes* in this data, other 11 bacteria belonging to other phyla. It has been previously shown that genera Lactobacillus, Pediococcus, Weisella, Lactococcus and Leuconostoc, all belonging to phylum *Firmicutes,* are linked to desirable lactic acid fermentation (Pahlow et al. 2003, Ogunade et al. 2018). These genera are also widely used in silage additives. In meta-analysis of Oliveira et al. (2017), 67 % of experimental silages were preserved with *Lactobacillus plantarum* and 27.3 % with a mixture of many lactic acid bacteria species.

In the present data genera *Pediococcus* and *Lactobacillus* are part of family *Lactobacillaceae* but not included to community of family *Lactobacillaceae* because only bacteria with unknown genus were viewed in family level. Still those three bacteria communities are close relatives to each other and that could explain some similarities in heatmaps. Genus *Enterococcus* belongs to other family of *Enterococcaceae* and family *Leuconostocaceae* was detected only in family level.

These 11 other bacteria followed a similar kind of fermentation profile. They preserved WSC and correlated negatively with total fermentation acids and total fermentation products. Aerobic stability and lactic and acetic acid were negatively correlated with those bacteria. Correlations to pH were significantly positive in most of those 11 bacteria, meaning that they prefer high pH and were not present in silages with low pH.

Family *Sphingobacteriaceae* and *Pedobacter* had negative correlation with ensiling losses and they were also negatively correlated with ammonia, which could mean that ensiling losses are related to protein degradation. Those bacteria which are related with low ensiling losses in silage also had connection with low amount of ammonia N. Strengths of the correlations were similar in both parameters within same bacteria. Genus *Enterococcus* was strongly positive with ammonia and ensiling losses and at the same time had negative correlation with DM. This means that genus *Enterococcus* increases protein and DM losses even though some species such as *Enterococcus faecium* are widely used as additives. Diversity within one genus can be remarkable and species-level information is needed when additive bacteria are selected.

Genus *Lactobacillus* had strong positive correlation to both acetic acid and aerobic stability. Some heterofermentative *Lactobacillus* species such as *Lactobacillus buchneri* are used as silage additives, because they can produce some acetic acid to silage to improve aerobic stability (Oude Elferink et al. 2001, Filya 2003).

Ogunade et al. (2018) scanned bacterial communities in high DM lucerne silage and found that genera *Sphingomonas* and *Stenotrophomonas* correlated with fermentation quality parameters in a way which is good for ensiling. Both genera had negative correlation with silage pH, ammonia-N and growth of moulds and yeasts. In present data, *Shingomonas* correlated positively with silage pH (0.69) and negatively with aerobic stability (-0.69), which is the opposite what Ogunade et al. (2018) found. *Stenotrophomonas* correlated also positively with silage pH (0.41) and negatively with aerobic stability (-0.26).

Some differences were observed between abundance of genus *Lactobacillus* and family *Lactobacillaceae*. Genus *Lactobacillus* is part of family *Lactobacillaceae*, but it was identified separately in this data. Genus *Lactobacillus* dominated soil-contaminated Control and Salt and was one of the dominant in tight non-contaminated with Salt. In other silages family *Lactobacillaceae* were the dominating bacteria together with genus *Shingomonas* (except for FA treatment in loose non-contaminated and tight contaminated treatments). *Lactobacillaceae* family is often the dominant taxa in silage and genus *Lactobacillus* includes most of it. In study of Eikmeyer et al.

(2013) *Lactobacillus* covered 29 % of all operational taxonomic units (OTU: taxonomical classification for close relative microbes, a cluster of organisms with similar gene marker in DNA sequencing) in untreated grass silage after 58 days of fermentation. In contaminated silages *Lactobacillus* was even more dominant (67% after 58 days).

Firmicutes were different from other bacteria not only in fermentation quality but also in bacterial interaction correlations. Genera *Enterococcus*, *Lactobacillus* and *Pediococcus* with families *Leuconostocaceae* and *Lactobacillaceae* had negative or weak correlation with other 11 bacteria on a same time when those 11 other bacteria correlated positively and significantly with each other (Figure 4). This underlines the conclusion that those five bacteria had different role in fermentation than other 11 bacteria.

Positive correlations within *Firmicutes* (*Enterococcus* to *Lactobacillus*, family *Lactobacillaceae* to *Pediococcus* and family *Leuconostocaceae* to *Enterococcus* and to *Lactobacillus*) could mean that competition between those bacteria is not strong and similar growing conditions in silage generate the positive correlation between them. There is a chance of some positive straight interactions between bacteria.

Those 11 other bacteria correlated positively with abundance of each other and negatively with most of the *Firmicutes*. Most likely these bacteria are related to weak lactic acid fermentation and high pH of silage. When Firmicutes caused strong lactic acid fermentation, those other bacteria declined respectively. It is uncertain do they just prefer high pH, or do they increase the pH with their metabolites.

Correlations between bacteria genera revealed some information about how bacteria interacted in silage. Bacteria which use same substrates and prefer similar pH competed against each other. Family *Lactobacillaceae* and genus Lactobacillus had negative correlation ($r=-0.45$, $P<0.01$) because they are so close to each other and they probably compete for same resources.

Some strong correlations such as between genera *Devosia* and *Agrobacterium* could be explained with pH they tolerate. Some silages had higher pH than others and bacteria which were present together in high pH, correlated strongly to one another. It is uncertain if interactions exist between two bacteria or do they just prefer or avoid similar circumstances in silage.

Associations between silage quality parameters and silage bacteria could be used when new silage additives are investigated. It shows the role of the most abundant bacterial populations on the fermentation process of silage and which bacteria have the greatest impact to each fermentation parameter. More detailed knowledge is needed all the way to species-level to select new bacterial silage additives. Information can help us to avoid undesirable microbes by using those which have strong negative correlation against unwanted microbe. More information is needed about microbiome changes in silage made in different forages e.g. legumes.

8 Conclusions

Main goals of this study were to investigate how ensiling management factors such as additives, compaction and hygiene affect the fermentation quality and bacterial population of silage. The study focused on bacterial communities and how they change during fermentation. Some interesting correlations between bacteria taxa and fermentation parameter were found. However, bacteria within same genus can be different from each other and correlations do not necessarily mean direct impact, so results are just guiding.

Tight compaction improved the silage quality by resulting on average lower pH and ethanol concentration and higher amount of lactic acid compared to loose compaction, but effect was clear only in CONT and SALT silages. Soil contamination had even bigger effect on fermentation than compaction level. It led to an extensive wild type of fermentation, which increased lactic and acetic acid concentrations, total fermentation products and VFAs but surprisingly decreased ensiling losses and improved aerobic stability on average when compared to non-contaminated silage. Longer aerobic stability was probably due high concentration of acetic acid in soil contaminated CONT and SALT silages. Anyhow wild type of fermentation is difficult to control, and it needs to

be avoided on farm level. Wild type fermentation can lead to nutrient losses, health problems to animals and hygienic risks in the food chain.

The 16 most abundant bacteria were selected to closer evaluation. Bacteria belonging to phylum *Firmicutes* and class *Bacilli* (genera *Pediococcus, Enterococcus* and *Lactobacillus* and families *Leuconostocaceae* and *Lactobacillaceae*) were clearly different from other bacteria and they led the ensiling process towards desirable lactic acid fermentation. Still *Enterococcus* and family *Leuconostocaceae* both had positive correlation to ammonia and ensiling losses which are signs of undesirable fermentation. Other 11 selected bacteria correlated negatively with fermentation products and lactic acid, but positively with high pH and residual WSC.

Formic acid based additive produced diverse bacterial population in silage, probably because not any single bacteria species was able to dominate the restricted fermentation process. Total amount of bacteria was most likely smaller in FA silages than in other silages, but population was more diverse. LAB treatment stimulated *Lactobacillaceae* family, even though LAB inoculant contained two strains of *Lactobacillus plantarum*, which should be part of genus *Lactobacillus* instead. In the present experiment, Salt additive was not particularly efficient to control fermentation and fermentation pattern and abundances of bacteria were closer to CONT than to LAB or FA silages.

All the additive types were able to improve the quality of non-contaminated silages compared to CONT silages. Soil contaminated CONT and SALT silages were extensively fermented and still stable because of too high amount of acetic acid. Too high amount of VFA and lactic acid and low amount of residual WSC decreased the nutritional value of those silages.

Tight compaction increased the abundance of both *Pediococcus* and *Lactobacillus* and decreased the abundance of *Agrobacterium* compared to loose compaction. Soil contamination had a remarkable impact on bacterial abundances, and it increased the abundance of *Lactobacillus* when abundances of other bacteria decreased. Genus *Lactobacillus* became clearly dominant in soil contaminated CONT and SALT silages, whereas abundance of family *Lactobacillaceae* declined.

Based on these outcomes forage should be ensiled with tight compaction and without any soil contamination to minimize risks for the fermentation process. Use of silage additives is strongly recommended. In this experiment both LAB and FA were effective, resulting in good quality silage. DNA sequencing can offer specific information about fermentation and microbiome of silage. Bacteria belonging to *Firmicutes* are responsible for suitable lactic acid fermentation in silage. In future research about different metabolites of silage microbiome is needed to achieve detailed knowledge of silage fermentation and it should be done with various forages and climate conditions. However, combining microbiome, metabolome and fermentation quality data is a demanding goal but it can lead to practical applications in silage preserving.

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Appendices

Appendix 1. Abundance of silage bacterial populations of grass silage treated with different additives under two compaction (Comp.) and soil contamination (Cont.) levels and real abundances of selected 16 bacteria in total identified bacteria

Contamination		Non-contaminated								Soil contaminated				SEM[2]	P-value[3]	
Compaction		Loose				Tight				Tight						
Additive	FM[1]	Control	FA	LAB	Salt	Control	FA	LAB	Salt	Control	FA	LAB	Salt		Comp	Cont
g_Cryocola	0.47	2.45^{b}	3.84^{a}	1.27^{c}	2.63^{b}	2.80^{b}	3.82^{a}	1.33^{c}	2.26^{b}	0.42^{d}	4.07^{a}	1.09^{cd}	0.49^{cd}	0.164	0.96	<0.01
f_Sphingobacteriaceae	0.38	0.99^{bc}	2.14^{a}	0.95^{bc}	1.03^{bc}	1.24^{b}	2.09^{a}	1.11^{bc}	0.96^{bc}	0.38^{c}	2.70^{a}	0.87^{bc}	0.41^{c}	0.155	0.53	0.10
g_Pedobacter	0.67	1.34^{bcd}	2.17^{ab}	1.34^{bcd}	1.28^{bcde}	1.54^{bc}	2.22^{ab}	1.54^{bc}	1.11^{cde}	0.36^{e}	2.87^{a}	1.07^{cde}	0.47^{de}	0.184	0.60	0.02
g_Enterococcus	0.00	1.55^{b}	0.08^{c}	0.01^{c}	2.85^{a}	2.25^{ab}	0.12^{c}	0.01^{c}	2.76^{a}	2.18^{ab}	0.07^{c}	0.23^{c}	1.98^{ab}	0.205	0.28	0.95
f_Lactobacillaceae	0.03	11.66^{bcd}	2.76^{cd}	57.81^{a}	18.93^{b}	15.63^{bc}	9.78^{bcd}	59.66^{a}	14.81^{bcd}	0.19^{d}	0.83^{cd}	61.61^{a}	0.16^{d}	2.945	0.31	<0.01
g_Lactobacillus	0.01	7.24^{c}	2.24^{c}	0.27^{c}	4.89^{c}	4.62^{c}	0.94^{c}	0.26^{c}	18.80^{b}	83.12^{a}	4.89^{c}	2.49^{c}	83.30^{a}	1.501	0.03	<0.01
g_Pediococcus	0.01	2.16^{bcd}	1.17^{dc}	6.03^{a}	4.54^{abc}	4.93^{ab}	1.49^{bcd}	6.42	7.30^{a}	0.28^{d}	1.30^{dc}	6.62^{a}	0.38^{d}	0.702	<0.01	0.01
f_Leuconostocaceae	0.00	2.08^{ab}	0.58^{ab}	0.00^{b}	1.98^{ab}	0.82^{ab}	0.12^{b}	0.01^{b}	2.88^{ab}	2.77^{ab}	0.06^{b}	1.32^{ab}	3.34^{a}	0.592	0.63	0.10
g_Mycoplana	0.14	1.71^{bc}	2.12^{ab}	0.98^{de}	1.70^{bc}	1.91^{bc}	2.10^{ab}	0.92^{de}	1.43^{cd}	0.33^{f}	2.61^{a}	0.65^{ef}	0.31^{f}	0.106	0.64	<0.01
f_Aurantimonadacea	0.14	1.88^{ab}	1.13^{cd}	0.33^{e}	1.76^{ab}	2.06^{a}	0.96^{d}	0.39^{e}	1.49^{bc}	0.18^{e}	1.20^{cd}	0.28^{e}	0.18^{e}	0.099	0.47	<0.01
g_Devosia	0.19	2.28^{a}	1.75^{a}	0.69^{b}	2.06^{a}	2.33^{a}	1.75^{a}	0.64^{b}	1.85^{a}	0.23^{b}	2.09^{a}	0.51^{b}	0.21^{b}	0.118	0.55	<0.01
g_Agrobacterium	0.17	2.18^{a}	1.45^{c}	0.64	2.09^{ab}	2.17^{a}	1.43^{c}	0.55^{de}	1.59^{c}	0.18^{e}	1.68^{bc}	0.48^{de}	0.19^{de}	0.089	0.02	<0.01
g_Rhizobium	0.14	1.33^{a}	1.38^{a}	0.33^{b}	1.50^{a}	1.56^{a}	1.45^{a}	0.33^{b}	1.25^{a}	0.12^{b}	1.57^{a}	0.29^{b}	0.12^{b}	0.102	0.85	<0.01
g_Sphingomonas	1.89	16.61^{ab}	19.46^{a}	9.02^{cd}	16.16^{ab}	18.05^{ab}	17.93^{ab}	9.41^{cd}	13.57^{bc}	3.67^{e}	20.27^{a}	6.83^{de}	3.71^{e}	0.954	0.40	<0.01
f_Comamonadaceae	0.26	1.33^{a}	1.20^{a}	0.66^{b}	1.47^{a}	1.53^{a}	1.14^{a}	0.62^{b}	1.21^{a}	0.17^{c}	1.23^{a}	0.59^{b}	0.18^{c}	0.079	0.52	<0.01
g_Stenotrophomonas	1.31	3.11^{bcde}	6.74^{a}	0.67^{de}	3.66^{abcd}	2.54^{cde}	6.27^{ab}	0.68^{de}	1.05^{de}	0.35^{de}	5.51^{abc}	0.51^{de}	0.24^{e}	0.664	0.07	<0.01

Values with same letter in a row are not significantly different at 5% Tukey test.
[1]Fresh matter before ensiling. [2] Standard error of the mean. [3]Effects of compactions and soil contamination.