Quantitative polymerase chain reaction method compared with traditional methods for *Clostridium tyrobutyricum* in clover-grass silage

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Abstract

C. tyrobutyricum is the most common type of bacteria to contaminate milk and cause late blown cheese caused by high spore counts in silage. The purpose of this thesis was to test a qPCR method to quantify *C. tyrobutyricum* in silage, by investigating potential differences in the clostridia spore estimate between the qPCR method, the MPN method and the plate count method. Furthermore, relationships between silage fermentation characteristics and clostridia spore contents analysed with the different methods were evaluated. The silage that was used was from a sward containing 75% red clover and 25% grass that was harvested on the 4th of September 2011 (third harvest) at Skara in the southwest of Sweden. The silage fermentation parameters were analyzed and the *C. tyrobutyricum* count was analysed with Qauntitative Polymerase Chain Reaction (qPCR method), most probable number method (MPN-method) and plate count method. Relationships between clostridia spore counts from the traditional laboratory methods (the plate method and the MPN method) and the qPCR method were investigated by linear regression analysis using PROC REG in SAS Also, the least-square means of clostridia spore counts from the analytical methods were compared in the general linear model procedure of SAS (PROC GLM). Stepwise regressions on the relationships between silage fermentation characteristics and clostridia spore count from the different methods were performed. Comparisons of the least-square (LS) means of clostridia spore count from the different methods did not show any significant differences between the methods. The simple linear regressions showed that the MPN method performed at the laboratory of SLU, Skara, Sweden (MPN-S) and the plate method had moderate to strong correlations with the qPCR method, which indicates their reliability for estimating the *Clostridium* spore content in clover-grass silage. The MPN method performed at the LKS mbH, Germany (MPN-D) showed low correlations with the qPCR. The qPCR method showed very high correlation with the fermentation parameters compared to the MPN-D and the plate count methods. Generally, the qPCR method hade higher correlation with the fermentation parameters and smaller variations compared with MPN and plate-method. Conclusion of the study was no significant differences in Clostridia spore counts of red clover-grass silage between the qPCR, MPN-D, MPN-S and the plate methods when comparing the LS means of the Clostridia counts from the different methods. The simple linear regressions showed that the MPN-S and the plate method had moderate to strong correlations with the qPCR method. The MPN-D showed low correlations with the qPCR. The high specificity for the qPCR method decreases the risk of counting other lactate degraders than *C. tyrobutyricum* in the silage. The qPCR method showed very high correlation with the fermentation parameters compared to the MPN-D and the plate count methods. The MPN-S method showed promising relationships with the fermentation products in the silage.

Sammanfattning

C. tyrobutyricum är den vanligaste bakterien till att kontaminera mjölk och orsaka sprängda ostar orsakat av höga sporhalter i ensilaget. Syftet med denna studie var att testa qPCR metoden för kvantifiering av *C. tyrobutyricum* i ensilage, genom att undersöka potentiella skillnader i den uppskattade mängden klostridiesporer mellan qPCR, MPN metod och platt metoden. Vidare undersöktes samband mellan ensilagets fermentationsparametrar och mängden klostridiesporer analyserade med de olika metoderna. Ensilaget som användes i undersökningen kommer från en vall som innehåller 75 % rödklöver och 25 % gräs som skördades den 4:e September 2011 (tredje skörden) i Skara i sydvästra Sverige. Ensilagets fermentationsparametrar analyserades och *C. tyrobutyricum* mängden analyserades med kvantitativ polymerasekedjereaktion (qPCR metoden), mest sannolika antalmetoden (MPN-metoden) och plattspridningsmetoden. Relationen mellan mängden klostridiesporer härrört ur de traditionella laboratoriemetoderna (plattmetoden och MPN-metoden) och qPCR-metoden analyserades i linjär regressionsmodell med PROC REG i SAS. Även jämförelser av medelvärden för klostridiespormängd mellan de olika metoderna utfördes i generell linjärmodell i SAS (PROC GLM). Stegvis regressionsanalys mellan fermentationsparametrar och klostridiespormängden från var och en av metoderna genomfördes. Det påvisades inga signifikanta skillnader i LS mean klostridiespormängd från de olika metoderna. De enkla linjära regressionerna visade att MPN-metoden som utförts vid laboratoriet i SLU, Skara, Sverige (MPN-S) och plattmetoden hade måttliga till starka korrelationer med qPCR-metoden, vilket indikerar deras tillförlitlighet för att uppskatta *Klostridium* sporinnehållet i klöver-gräs ensilage. MPN-metoden utförd vid LKS mbH, Tyskland (MPN-D) visade låga korrelationer med qPCR. QPCR-metoden visade mycket hög korrelation med fermentationsparametrarna jämfört med MPN-D- och platträkningsmetoden. Generellt hade qPCR-metoden högre korrelation med fermentationsparametrarna och mindre variationer jämfört med MPN- och plattmetoden. Slutsatsen av studien var, analysen där LS mean jämfördes mellan metoderna gav ingen signifikant skillnad i *klostridium* mängd mellan qPCR, MPN-S, MPN-D och plattspridnigsmetoden i klöver-gräs ensilage. Enkla linjära regressioner visade att MPN-S och Platt metoden hade starka korrelationer till qPCR metoden. MPN-D visar låg korrelation med qPCR Specificiteten hos qPCR metoden minskar risken att andra laktat fermenterare medräknas. qPCR- metoden visade stark korrelation med fermentationsparametrarna i jämförelse med MPN-D och plattspridningsmetoden. Även MPN-S visade korrelation med fermentationsparametrarna.

Table of contents

THANKS TO....3
ABSTRACT....4
SAMMANFATTNING....5
INTRODUCTION....8
AIM....9
LITERATURE REVIEW....10
Silage....10
Clostridium....11
Bacillus....14
Silage additives....15
Methods to detect *C.tyrobutyricum* in silage....16
MATERIALS AND METHODS....21
Collection of samples....21
Analysis of the silage....22
Statistical analyses....27
RESULTS....29
Comparisons of *C. tyrobutyricum* analysis methods....29
Relationships between *C. tyrobutyricum* count and fermentation characteristics of the silage for each method....31
DISCUSSION....38
Comparisons of *C. tyrobutyricum* analysis methods....38
Relationships between Clostridium count and fermentation characteristics of the clover-grass silage for each method....40

CONCLUSIONS 44

REFERENCES 45

Introduction

Clostridium spp. is an obligate anaerobic and gram-positive bacteria that occurs naturally in soil (Breed, Murray & Smith, 1957). *C. tyrobutyricum* is the most common type to contaminate the milk and cause late blown cheese (Garde *et al*., 2011: Feligini *et al.,* 2014). Dairy companies have reported problems with these spores in milk, which have led to that comprehensive research has been done, particularly in feed production and stable hygiene (Andersson *et al*., 2006: Driehuis, 2013).

The two most commonly used methods for analysis of clostridia in silage are most probable number method (MPN-method) and plate count method. There are also a few reported analyses by quantitative **P**olymerase **C**hain **R**eaction (qPCR method) for clostridia in silage (Bassi *et al*., 2013; König *et al*. 2017). Analysis for clostridium spores in silage and milk is mainly done by the MPN-method (Garde *et al*., 2011: Driehuis, 2013: Zucali *et al*., 2014). Today, the MPN method with Bryant Burkey broth (BBB) medium is used in Sweden (Andersson *et al*., 2008). One of the problems with this method is to separate the different species of clostridium (Cato *et al*., 1986). The variation in results between different methods and different laboratories is a problem as well as the fact that different types of cheeses tolerate different amounts of spores before late blowing. This makes it difficult to create general guidelines for the amount of spores in milk or in silage, which will decrease the risks for late blown cheese (Cremonesi *et al*., 2012: Zucali *et al*., 2014).

The plate count method is commonly used in research experiments, since both quantity and species (after some confirmation tests) can be determined. For this analysis of clostridium, the Reinforced Clostridia Medium (RCM-medium) is often used (Jonsson, 1990). However, this plate count method is not used, for example, by dairy industry because it is more resource and time consuming (about 7 days) than the MPN-methods (SVA, 2014).
The PCR method was invented by Kary B. Mullis in the 1980s (Nobelprize, 1993) and developed for quantification of clostridia analyses in silage (Bassi *et al*., 2013). The problem is to find efficient methods to extract the clostridia DNA and to handle the PCR inhibitory substances (for example enzymes) that could interfere with the PCR-reaction (Cremonesi *et al*., 2012; SVA, 2014).

Clostridium spores are a problem for dairies, and for the farmers. Growth of clostridia in silage reduces the feed value, the palatability and a low number of it in the milk can give a price reduction of the milk. The price reduction depends on the type of cheese production (Andersson *et al*., 2008). A fast, cheap, easy, and reliable method to determine the content of clostridium spores in silage would therefore facilitate both dairies and farmers.

Aim

The purpose of this thesis was to test a qPCR method to quantify *C. tyrobutyricum* in silage, by investigating potential differences in the clostridia spore estimate between the qPCR method, the MPN method and the plate count method. Furthermore, relationships between silage fermentation characteristics and clostridia spore contents analysed with the different methods were evaluated to investigate if the clostridia spore content can be made from silage fermentation end products.

Literature review

Silage

Silage is forage preserved by limited water activity in an anaerobic environment (McDonald, 1982; Kung, 2001; Driehuis, 2013) and low pH-value (Carvalho *et al*., 2014). There are many different crops that can be ensiled. In Sweden it is common to use grass, e.g. timothy (*Phleum pratense* L.), meadow fescue (*Festuca pratensis* Huds.), and cocksfoot (*Dactylis glomerata* L.) mixed with legumes, e.g. clover (*Trifolium spp.*) as roughage (Weidow, 2000). Water activity is defined as availability of water and is a requirement for growth of prokaryote and eukaryote organisms. In order to reduce the water activity in the silage, crops usually are wilted in the field (Muck *et al*., 2003). To prevent clostridia growth, wilting should be done to a dry matter (DM) content of 25-30% (Wieringa, 1958; Kung, 2001).

When the crop has been wilted, the bales will be made and wrapped in plastic. The wrapping is done in order to create and maintain an anaerobic environment, naturally developed created when the plants and microorganisms respire (McDonald *et al*., 2002). The depletion of oxygen will inhibit the respiration, which stops when there is no oxygen available (Wilkinson & Davies, 2012). In bunker silos, packing of the forage between loads of forage and sealing with plastic cover on top of the silo and along the sides is important for creating a lasting anaerobic environment.

When the oxygen is depleted, the fermentative bacteria (initially acetic acid bacteria) will start to grow. They use water soluble carbohydrates (WSC) and produce acetic acid, ethanol and some lactic acid (Schroeder, 2004). This production of mainly acetic acid will cause the pH- value to decrease. Usually well-ensiled silage contains less than 0.5% alcohol of DM (Carvalho *et al*., 2014) and less than 1-3% acetic acid of DM (Seglar, 2003). If the bales do not maintain anaerobic environment, the alcohol values could be higher (Kung & Shaver, 2000). Yeast growth is the main reason for high alcohol concentrations. Yeast consumes WSC and produce mainly ethanol but also propanol, butanol, pentanol (Kyuei *et al*. 1977). Yeast can also respire by use of lactic acids, which lead to an increased pH-value of the silage (Pahlow *et al*., 2003). The acetic acid bacteria will stop growing when the pH-value is approaching 5. Instead the lactic acid bacteria (LAB) will start growing (McDonald, 1982). Lactic acid bacteria produce lactic acid and silage normally contains 3-6% lactic acid of DM but it varies with the DM content and particle length of the forage (Seglar, 2003). The low pH-value (<4) inhibits the growth of clostridia (Henderson & McDonald, 1975), by destroying the plasma membrane of the cell and inhibits enzymes due to osmosis (Willey *et al*., 2009). If the silage do not maintain the low pH- value, the silage will risk suffer from rapid deterioration. If the pH-value is high (<5) and the lactic acid concentration is low (<65% of total acids in the silage) it can be owing to clostridia growth, Bacillus, yeast or high DM (>50% for bal) (Kung & Shaver, 2000: Kung, 2001). Also, propionic acid can be formed in the silage, which usually comprises less than 1% of DM of the silage (Seglar, 2003).

Clostridium

Clostridium spp. is a rod shaped, strictly anaerobic, gram-positive bacteria that occurs naturally in soil (Pahlow *et al*., 2003). Fresh crops in the field are growing in the soil and can therefore contain up to 100-1000 colony – forming units (cfu) of butyric acid bacteria spores (BAB)/g fresh crop (Pahlow *et al*., 2003; Visser *et al*., 2007). Strict anaerobic means that the bacteria only can metabolize and grow in an environment without oxygen (O_2) or at a very low level (Redox potential control -320) (Jonsson, 1989; Jonsson, 1991; Pahlow *et al*., 2003; Liu *et al*., 2013). Visser *et al*. (2007) observed that silage often contained high amounts of spores (10^5 CFU/g) on the surfaces where oxygen could penetrate. These Gram-positive bacteria can form endospores and reproduction occurs by binary fusion (Willey *et al*., 2009). Spore-forming occurs for example when the bacterium is exposed to different stresses for example temperature, lack of nutrient or water or by quorum sensing systems (signal system in bacteria which works by signal molecules that activate the spore-forming gen) (Kotte *et al*., 2017). The spores can survive boiling temperatures, normal pasteurization, low temperatures, no available nutrients and chemical disinfections. Therefore, these spores are a major problem for the dairy (Garde *et al*., 2010; Driehuis, 2012).

Clostridia are divided into proteolytic and saccharolytic bacteria, where the proteolytic bacteria ferments amino acids and saccharolytic bacteria ferments soluble carbohydrates and organic acids, typically lactic acid (Pahlow *et al*. 2003). *C. tyrobuyricum* belongs to the saccharolytic bacteria and break down the lactic acid in two steps. The *C. tyrobuyricum* fermentation can be described as following:

Lactic acid (3 C) + acetic acid (2C) → butyric acid (4C) +CO_2

Lactic acid (3C) → acetic acid (2C) + CO_2+ $2H_2$

The two reactions can be summarized in to following reaction:

2 Lactic acid or Glucose (6C) → 1butyric acid (4 C) + $2CO_2$ + $2H_2$

(Rooke & Hatfield, 2003; Zhu & Yang, 2004).

Clostridia fermentation in the silage will result in DM losses, energy losses and the pH-value in the silage will increase. The DM loss and the energy loss will be due to the production of carbon dioxide and hydrogen (Bryant, Burkey, 1956). The energy losses will be bigger in the silage when acetic acid are produced due to more adenosine triphosphate (ATP) will be produced. The production of acetic acid occurs by substrate level phosphorylation which requires electron acceptor for example butyrate (Thauer, Jungermann, Decker, 1997). The pH will increase since 2 moles of lactic acid will be fermented to the weaker acid, 1 mole of butyric acid (Bryant, Burkey, 1956). The pH in the silage will determine which byproduct that will be dominantly produced. If the pH is about 6, butyric acid will be the dominant product and if the pH is about 5, acetic acid will be the dominant product produced. Pyruvate will be produced from lactic acid by independent nikotinamidadenindinukleotid actate dehydrogenase. From pyruvate pyruvate-ferredoxin oxidoreductase acetyl-CoA will be formed, depending on pH. Phosphotransacetylase will be activated forming acyl phosphates or acetyl-CoAacetyltransferase will be activated and produce butyryl-CoA. Acyl phosphates will be catalyzed by acetate kinase to acetic acid

and adenosintrifosfat. Butyryl-CoA form acetyl phoshate by phosphotransbutyrylase and butyrate kinase catalyzes acetyl phoshate to butyric acid (Zhu & Yang, 2004).

Clostridium in silage

When the crop is fertilized and harvested, it may become contaminated by clostridia. The amount varies depending on the number of spores in the soil and if manure has been spread on growing crop (Pahlow *et al*., 2003). Contamination of soil in the fresh matter increases the amount of spores in silage and therefore the farmer has to be careful to correctly adjust the machines (depends on type of machine) and avoid driving with muddy tires in the crop and in silos (often high number so spores close to bunker silos). The risk of clostridia growth in the silage increases if the fresh matter has high temperature, low WSC content (Gibson, 1965), low DM (<25% DM) content, a leaky silo and plants that have a high buffering capacity such as clover (Gibson, 1965). There is a greater risk for clostridia growth when the silage is exposed to an aerobic leakage, which favors yeast growth. The yeast creates an anaerobic environment but consumes the lactic acid which creates perfect conditions with an elevated pH for the clostridia to thrive (Beynum & Pette, 1935; Jonsson, 1989: Jonsson, 1991; Vissers *et al.* 2007; Tabacco *et al*. 2009). The yeast produces ethanol in an anaerobic environment. Yeast also ferments glucose to VFA (propionate, butyrate, acetate), carbon dioxide and propanol (Middelhoven & van Baalen, 1988). Jonsson (1990) also observed a higher level of *C. tyrobutyricum* on the surface of the silage bale where the pH-value was higher compared to deeper inside the silage.

The main problem with *C. tyrobutyricum* in silage is that it consumes lactic acid so that the pH value increases in the silage (Van Beynum & Pette, 1935). Butyric acid is normally less than 0.1 % of fresh silage (Seglar, 2003: Carvalho *et al*., 2014) and ammonia-nitrogen (N) concentrations less than 8% of total N (< 80 g / kg N) is desireable (Åkerlind, 2009). High butyric acid concentrations (> 0.1% of fresh silage) give a characteristic butyric smell in the silage. High ammonia concentrations gives a characteristic ammonia smell in the silage (McDonald, 1982).

Clostridium effect on animal

Growth of clostridia in silage can decrease the production and give poor animal health (Kung *et al*., 2000). High concentrations of ammonia and butyric acid in the silage (*C. tyrobutyricum* growth) can result in decreased feed intake in dairy cows and thereby cause decreased production (Gill *et al*., 1988; Kung *et al*., 2000). A study by Krizsan and Randby (2006) showed that butyric acid was well correlated with silage dry matter intake (SDMI).They concluded that prediction of SDMI could be improved, by including individual acids for example butyric acid, instead of total acids produced in the silage. Krizan *et al*. (2007) observed that silage with increased NH_3-N reduced feed intake in cattle. The result also showed that increase butyric acid in silage to growing cattle did not affect the DM intake. Another Clostridium bacteria, *C. botulinum* can produce toxin that acts as a neurotoxin. The neurotoxin can lead to death of animals (Sugiyama, 1980: Cobb *et al*., 2002).

Clostridium in milk

Growth of *C. tyrobutyricum* is a problem in silage but also in milk, since *C. tyrobutyricum* can cause late blown cheeses in cheese manufacturing (Klijn *et al*., 1995). *C. butyricum, C. sporogenes* and *C. beijerinckii* can also cause late blown cheeses (Cocolin *et al*., 2004: Garde *et al*., 2011). The “late blown cheese” problem affects hard cheese especially round

squeaky (Greve, Jarlsberg, Gouda, Emmentaler) and is recognized as large holes in the cheese and off flavoure. This defect is due to the *C. tyrobutyricum* bacteria. Instead of forming both D- and L-lactate as under normal cheese fermentation, Clostridium will form butyrate and hydrogen. The hydrogen gas causes big holes in the cheese texture (Mcsweeney, 2004). Huchet *et al*., (1997) observed that *C. tyrobutyricum* uses both the D- and the L-form from the lactic acid but prefer the D-form.

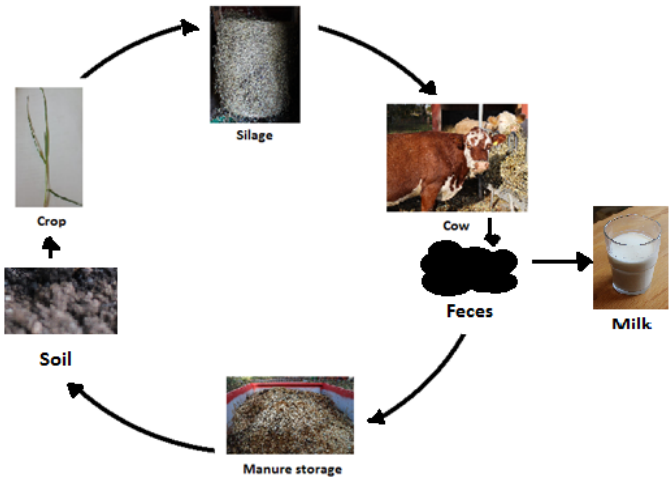

Figure 1. Contamination of clostridia from farm to dairy (modified from Pahlow *et al.*, 2003).

When contaminated silage is fed to dairy cows, the spores pass on through the gastro intestinal tract and end up in the faeces. The manure contaminates the teats and during milking the milk gets contaminated from the dirty teats (Pahlow *et al*., 2003;Nadeau *et al*., 2010; Arnesson *et al*., 2011) (Figure 1). In a study by the Swedish Dairy Association it was observed that dirty animals lead to 3-5 times higher amounts of spores in the bulk milk (Christiansson, 2009). In a study by Arnesson *et al*. (2011) 23 farms in Sweden were observed, half with high amount of spores (> 600 spores/l milk) in the milk and the other half with low amount of spores (<600 spores/l milk). Farms were also grouped depending on the amount of manure that they used (20-50 ton/ha and season, 60-80 ton/ha and season, 90-110 ton/ha and season). Samples were taken from manure, soil, crop, straw, silage and milk. Also interviews with farmers was done. In the study they concluded that problems with high amount of spores in milk started in the silage and that it required good silage hygiene. The problem increased with dirty cows. With good management and hygiene from silage to milk, problems with spores can be avoided (Arnesson *et al*. 2011).

When the cheese is stored, the spores get activated due to enabling environment (< 25% DM, anaerob (by wax), pH > 4 and the clostridia bacteria start to grow in the cheese (Visser *et al*. 2006). Julien *et al.* (2008) studied the sources of clostridia spores in milk. The soil, silage and milk were analyzed using RCM and PCR. Soil contained such high levels of clostridia spores that it exceeded the maximum limit of detection (0.72 CFU/g). Hay and silage stayed between the limits of detection (0.30 – 0. 72 CFU/g), while milk samples contained a lower amount of spores than the minimum detection limit (0.30 CFU/g). When the results also were analyzed with PCR the results showed that all samples analyzed with PCR contained clostridia-DNA, which they believed indicated that clostridia is a part of the ecosystem in milk production. In the experiment of Visser *et al*. (2006), a

relationship between high levels of clostridium spores in silage and farm tanks was observed. The observations indicate that the silage with a spore content of >0.70 CFU/g should not be fed to dairy cows because it will give high spore concentrations in the farm tank milk. Nadeau *et al*. (2010) drew similar conclusions as Visser *et al*. (2006). Dairy cows fed silage containing high levels of clostridium spores usually get high levels of clostridium spores in milk. In another study, Klijn *et al*. (1995) observed that about 88% of Gouda cheeses that were contaminated with *C. tyrobutyricum* had butyric acid fermentation. Some *C. tyrobutyricum* strains (CNRZ 611, EFAM 1527, NIZO S46 and NIZO FL104) did not cause butyric acid fermentation in cheese. However, in all cheeses with distinct blown symptoms *C. tyrobutyricum* were detected by a PCR-analysis.

Spores at dairy

Dairies use Bactofuge or Bactocatch to remove clostridium spores in milk with an efficiency of approxemently <4 sporer/L. When using Bactofuge, the milk is centrifuged to separate the spores from the milk. The milk then gets heated and cooled. Bactocatch (TetraPak) is on the contrary based on micro-filtration (1.4 micron pore size) where the filtrate gets pasteurized (>120 °C) and cooled. The cost for bactofuging is about 0.20 SEK/kg milk and Bactocatch cost is about 0.30 SEK/kg milk. (Andersson *et al*., 2008).

Bacillus

Bacillus can often appear in silage with clostridia spores and is therefore relevant to shortly bring up in this thesis. In appearance *Bacillus* is a rod-like bacterium and just like clostridia they form endospores. Bacilli are found in soil (Pahlow *et al*., 2003). In the field, crops usually contain 100-1000 CFU /plant of *Bacillus* (Pahlow et al, 2003). Normal amounts of *Bacillus* spores in the silage is less than 10^5 CFU/g (Seglar, 2003).

Bacillus are aerobic (e.g. *B. sphaericu, B. lentus, B. firmus)* (McDonald *et al*., 1991: Simoes *et al*., 2010) or facultative anaerobe (e.g. *B. cereus, B. polymyxa, B. licheniformis*) (Cato *et al*., 1986: McDonald *et al*., 1991). In silage, *Bacillus* ferments organic acids (Cato *et al*., 1986), and since they can withstand low pH-values it is hard to inhibit bacilli growth in silage (Woolford, 1977). The facultative anaerobic *Bacillus* seems to favour grass silage (Gibson *et al*. 1961), high temperature (>40◦C) (Gibson *et al*., 1958; Lindgren *et al*., 1985) and surface of grass silage (Lindgren *et al*., 1985; Driehuis & Oude Elferink, 2000).

Most *Bacillus* species do not cause any harm in animals but might consume lactic acid in to produce acetic acid, bytyric acid lactic acid ethanol, glycerol or 2.3 butanediol (Claus & Berkeley, 1986) in the silage. Making the pH increase, which create favourable environment for other unwanted microorganisms (Driehuis *et al*., 2009). According to Woolford (1977) the *Bacillus* compete against the lactic acid bacteria about the carbon recourses, which can inhibit the lactic acid bacteria. Therefore *Bacillus* should be prevented in the silage. *Bacillus ssp* from the silage contaminates the milk, in the same way as clostridia. The spores contaminate the silage and pass the digestive track and end up in the faeces that contaminate the teat (Eneroth *et al*., 2001).

The problem with *Bacillus* spores in the milk is that some i.e. *B. cereus* can form dangerous toxins in the milk, which can cause food poisoning. *B. cereus* can also cause coagulation lumps and taint the milk (Livsmedelsverket, 2014). Some *Bacillus* species can cause false positive responses in the MPN test for clostridia (Andersson *et al*., 2008).

Östling and Lindgren (1990) observed that crops fertilised with manure had a 20-40 times higher amount of *Bacillus* spores than artificially fertilised crops. It has also been shown that dirty teats from manure were the main source to high *B. cereus* in the milk. But the spore amount in the roughage was too low to be a risk for contamination of the milk (Christiansson *et.al*., 1998). Christiansson and Svensson (2007) also concluded that silage had too low amount of *Bacillus* spores to make any harm in the milk.

To prevent *Bacillus* growth in the silage it is important that the pH-value is low, anaerobic conditions and not too low DM content (< 25%; Giffel *et al*., 2002).

Silage additives

Homofermentative lactic acid bacteria

Homofermentative bacteria are bacteria that from glucose produce lactic acid (Dairy Foods Science Notes, 2010). The inoculants of homofermentative LAB are best suited for silage preservation when problem with clostridia growth is expected to be the major problem, which is characterized by low WSC concentration in the green crop and relatively low DM (20-30%) in the produced silage (McDonald *et al*., 1991).

The general goal of adding a starter culture of homofermentive LAB e.g. *Lacto plantarum*, alone in grass/legume silage is to get a fast drop in the pH-value, high final concentration of lactic acid and hence less competition from unwanted microorganisms (Weinberg & Muck, 1996). Jonsson & Lindgren, (1991) showed that lactic acid (2% of DM) inhibits *C. tyrobutyricum* in silage at a pH-value of 5. The mechanism assumed is that homofermentative LAB rapidly ferment available glucose in the silage outcompeting the clostridia by consumption of the nutrients and production of inhibitory acids. Sang Buem *et al*. (2014) showed that the homofermentative LAB provides the silage with lactic acid and decreased DM losses of the silage during the storage.

Heterofermentative lactic acid bacteria

Heterofermentative bacteria are bacteria that consume glucose and the main products are lactic acid, carbon dioxide, acetic acid and ethanol (Dairy Foods Science Notes, 2010). The heterofermentative LAB, e.g. *L. buchneri*, manly ferment fructose to lactic acid and acetic acid in the silage, thereby resulting in higher DM losses during ensiling than heterofermentative LAB (Sims, 1968; Nadeau & Auerbach, 2014). Sang Buem *et al.* (2014) observed that by adding heterofermentative LAB to forage at ensiling, aerobic stability can be improved.

Combination of homofermentative and heterofermentative lactic acid bacteria

Combination of heterofermentative LAB and homofermentative LAB improve both aerobic stability and the fermentation process in grass silage (Filya *et al*., 2007). This is due to that heterofermentative LAB mainly produce acetic acid and homofermentative LAB produce lactic acid. Together they provide an additive with dual purpose inoculants (Nadeau & Auerbach, 2013). Driehuis *et al*. (2001) showed that by adding a combination of heterofermentative LAB and homofermentative LAB to grass silage, the aerobic stability and fermentation process could be improved.

Chemical additives

Salt additives are efficient both in grass and legume silages and in their mixtures. Sodium benzoate inhibits growth of mould and yeast in silage and acts best at low pH-values (Woolford, 1975). Sodium benzoate inhibits the organisms by destroying the plasma membrane by osmosis (Krebs *et al*., 1983). The osmosis will make the liquid to move with the concentration gradient (from lower concentration to higher). A hypertonic additive has a higher hydrogen ion concentration than the microorganism in the silage. Making the liquid in the microorganism cell move out of the cell, the cell will shrivel. A hypotonic additive will instead have lower concentration than the microorganism cells, making the liquid flows in to the cell and the cell will lyse (burst) (Willey *et al*., 2009).

Nitrite is very effective against clostridia (Wieringa, 1966; Spoelstra, 1985). Potassium sorbate is effective against mould, yeast and clostridia (Woolford, 1975). Potassium sorbate inhibits the unwanted microorganism by osmosis (Krebs *et al.,* 1983). Results by Nadeau and Auerbach (2014) showed that butyric acid production can be inhibited in red clover-grass silage by use of additives containing nitrite, hexamine, potassium sorbate and sodium benzoate. The silage additive lowered the pH and the DM losses in the silage. The results also showed that use of this additive type less ammonia and more lactic acid wear produced in the silage. Clostridia can also be inhibited by adding acids and their salts to the silage. A mixture of formic acid, sodium formate, propionic acid and benzoic acid is an effective inhibitor of butyric acid producing clostridia (Nadeau and Auerbach, 2014).

Knicky and Lingvall (2004) observed that silage quality could be improved by different chemical additives in the silage. A mixture of sodium benzoate and sodium bisulphite improved the aerobic stability and reduced the nutritional losses. The combination of sodium benzoate, sodium nitrite, hexamine, and sodium propionate inhibits clostridia growth in silage (Knicky and Lingvall, 2004). Knicky and Spörndly (2009) also found that sodium benzoate, sodium nitrite and potassium sorbate inhibit clostridia, yeast and mould in grass silage. By inhibiting these unwanted microorganisms the silage a lower pH and better storage stability is achieved.

Methods to detect *C. tyrobutyricum* in silage

DNA extraction and quantitative polymerase chain reaction method

The qPCR method may be used to quantify and identify microorganisms (Takeuchi *et al*., 1997: Klocke *et al*., 2006: Mancini *et al*., 2010), e.g for microbial analysis of silage (Cremonesi *et al*., 2012; Bassi *et al*., 2013). The qPCR method is based on quantification of DNA (Manit *et al*., 2005; Valasek & Repa 2005). Before performing the qPCR, the DNA has to be extracted (Bassie *et al.,* 2013). First step of the extraction procedure making the cell walls weak and facilitate releasing the DNA from the cells using special chemical substances, like enzymes e.g. lysozyme. The lysozyme is suitable to extract DNA from gram positive bacteria since the enzyme hydrolyses beta-1, 4 bonds in the peptidoglycan layer between N-acetylglucosamine and N-acetylmuramic acid in their cells walls (McKenzie & Withe, 1991). To facilitate the extraction of DNA, it is common to use a so called kit, which is a commercially available product with all necessary chemicals and filters for an efficient extraction of DNA.

To copy the DNA, the double stranded DNA has to be first split into two singlestrands, which is called denaturation and it is done by heating in 94 °C (Figure.2). When heated the two DNA strands are separated from each other (Valasek & Repo 2005; Manita *et al*., 2005). To get two new double stranded DNA molecules out of the two single strands, new parts for each single part of the DNA has to be synthesized. It starts with binding of the short matching DNA fragments called the primers. Primers are short synthetic oligonucleotide sequences specific for the exact organism (here: bacteria) that is going to be analysed. Primers are designed based on the available genome or gene sequences, which are stored in a data base available for all researchers i.e. the Genebank (https://www.ncbi.nlm.nih.gov/genbank/). Using special programs species-specific sequences are identified and the primer pairs matching to these sequences are designed (Cremonesi *et al*., 2011). Then the temperature is lowered to 54 °C to enable primers to find and bind to a matching DNA-sequence on the single-stranded DNA and this stage is called annealing. The primers are binded to the matching DNA sequences because the hydrogen bonds between nucleotides "get stronger" as the temperature is lowered. When primers bind to the single-stranded DNA the temperature is raised to72 °C, the polymerase binds and starts attaching the complementary nucleotides to the single-stranded DNA. Most common DNA polymerase is Taq polymerase from *Thermus aquaticus*, because it is heat resistant and has the property for repeatedly create new DNA strands using primers and a single-stranded DNA as a matrix. It is crucial that the DNA polymerase enzyme is heat resistant because it has to withstand high temperature during denaturation stage before each cycle of the PCR-analysis. After each amplification cycle, two double-stranded DNA molecules are created from one DNA molecule.

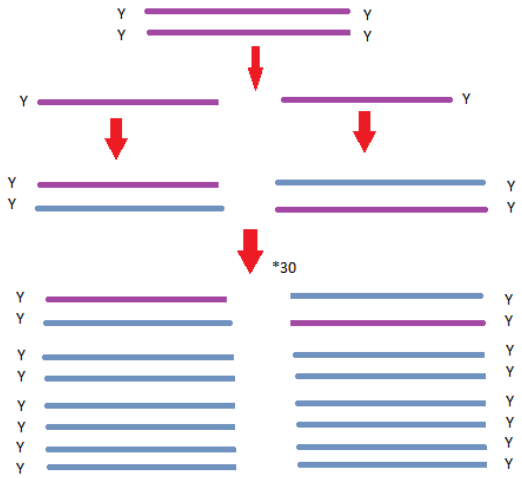

Figure 2. Copy of the DNA by PCR (modified from TaqMan GX, 2015).

To improve specificity of the qPCR reaction and to be able to quantify the number of DNA in the sample, a probe consisting of a reporter in one end and a quencher in the other binds to the ssDNA (single strained DNA) between the primers (Figure 3). Probes are designed in the same way as primers (see above). When the polymerization process of new DNA strand reach the 5' end of the probe, the reporter is removed and became fluorescent. The signal is recorded by a detector in the thermocycler and presented by the special software as a diagram of multiplication (Figure 4). The reporter signal is then suppressed by the quencher, when it is released by the polymerase in the similar way as the reporter.

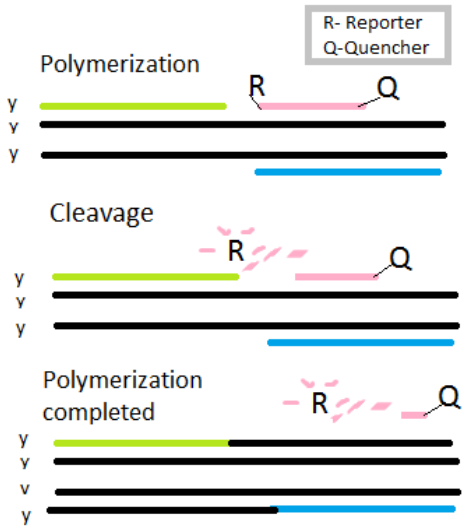

Figure 3. Function of probe (modified from TaqMan GX, 2015).

The plot consists of a baseline, fluorescent units (ΔRn), threshold and threshold cycle C_t (number of cycle at which the intensity of the fluorescence crosses the treshold) (Figure 4). The baseline represents the initial cycles of PCR in which there is little changse in fluorescence signal. When the specific DNA is present, the primers and probe bind to DNA and the fluorescence from probes increases, and if there is enough DNA it passes the "threshold value" (Ct) and is quantified. Ct is dependent on the amount of DNA, but Ct often is between the third and fifteenth PCR cycles. ΔR_n is R_n- baseline where R_n is the observed intensity of fluorescence during each cycle The Ct is always above the baseline and when the exponential phase of amplification begins is calculated by the special software (Manita *et al*., 2005).

The copying is exponential and there is a relation between the start amount of DNA and the cycles to reach detectable levels of DNA. This observation together with standard samples containing known amounts of DNA specific for the analysed species is used to quantifying the analysed organisms in the samples (Manita *et al*, 2005; Valasek & Repa, 2005).

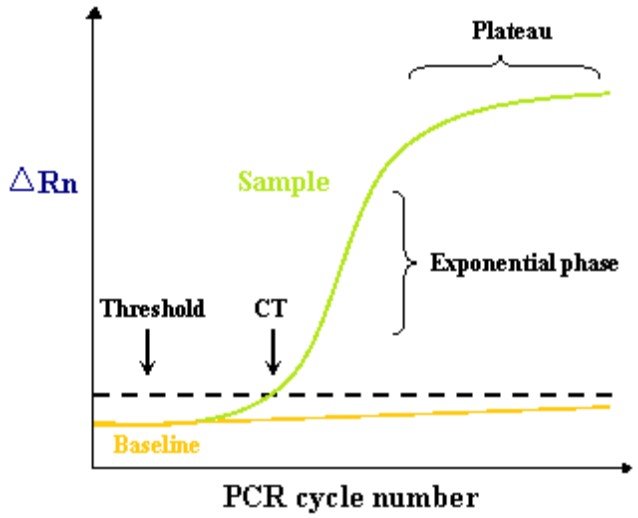

Figure 4. qPCR-plot (modified from Arya *et al*., 2005)

The use of PCR methodology has so far not been studied particularly intensively for silage microorganisms, but some PCR protocols for detecting and quantifying *Clostridia spp.* are known. In a study by Klijn *et al*. (1995) a PCR method to analyse *C. tyrobutyricum* in milk was used. They observed that the PCR method had an increased sensitivity to *C. tyrobutyricum* compared with the traditional methods. Furthermore, Enriquez *et al*. (2007) have developed a real-time PCR to quantify *C. tyrobutyricum* in milk. They observed that the method was 100% specific and may detect 25 spores in 25ml of milk. Cremonesi *et al*. (2012) studied the possibility of identifying *C. beijerinckii, C. butyricum, C. sporogene,* and *C. tyrobutyricum* with multiplex PCR assay from milk, hard cheese and silage. They observed that their method could distinguish between few clostridium strains. They also showed that the method had a good specificity and gave no false positive results. The specificity was at th level of 200 ng down to 0.78 ng of DNA corresponding to 102 cfu ml^{-1}. For *C. beijerinckii* specificity was down to 0.39 ng. Bassi *et al.* (2013) have made one of the few studies on silages in which they developed a TaqMan protocol for detecting and quantifying *C. tyrobutyricum* in silage. They used different clostridium strains and other bacteria (*Enterococcus, Lactobacillus, Streptococcus, Staphylococcus,* and *Bacillus*) to test the specificity of the primers and probe. They proved that the sequence of the selected phosphotransacetylase gene (pta-gene) is different from the sequences in any of the other bacteria which gives a method 100% specificity and allowed identification and quantification of *C .tyrobutyricum* with qPCR.With the method, as little as 14 bacteria ml^{-1} could be detected in a silage sample. They concluded that qPCR protocol together with the proposed DNA extraction method can be used to quantify *C. tyrobutyricum* and thereby quickly determine whether there is presence of *C. tyrobutyricum*. König *et al*. (2017) used qPCR method for analysing *C. tyrobutyricum* in lupin-wheat silage. The detection limit was 2000 gen copies per gram silage (200 bacteria g^{-1} silage) after using the Macherey-Nagel NucleoSpin Soil kit (MachereyNagel GmbH and Co. KG, D€uren, Germany) for extraction. The results showed that lupin-wheat silage treated with formic acid had a higher content *C. tyrobutyricum* than silage treated with sodium nitrite–hexamine mixture or homofermentative LAB. The results also showed that *C. tyrobutyricum* was the most abundant of the clostridia species in the silage.

The advantages of the PCR method are the accuracy, the short analysis time and that dead microorganisms also can be detected (Valasek & Repa 2005; Mancini *et al.*, 2010;

Cremonesi *et al*., 2012; SVA, 2014). The disadvantages are that the method still is expensive, sensitive to the quality of the DNA/RNA and removing of inhibitory substances e.g enzymes is difficult and expensive (Valasek & Repa 2005; SVA, 2014).

Most probable number method

The Most Probable Number (MPN) method is used to estimate the concentration of bacteria in a solution (Sutton, 2010). The method is based on calculating the most probable concentration of microorganisms in a sample. This is done through a series of tests in which registration of growth or not, in different environments, solutions and concentrations are done (Sutton, 2010). The results from the test used, is a series of numbers of positive tubes (for example 4 out of 5) at the different dilutions used from which the most probable concentration of microorganisms can be statistically calculated (FDA, 1978).
There are various substrates that might be used to estimate the presence of clostridia. The most commonly used substrate is the Bryant Burkey broth (BBB) substrate. This test is based on that clostridia can produce gas in anaerobic environment with lactate and acetate as the major carbon sources (Cocolin *et al*. 2004). Lactate-Acetate-Agar could also be used as substrate. Resazurin acts as a redox indicator (colour indicator) in the substrate (Cerf & Bergere, 1968)**.**

In an experiment performed by Ingham *et al*. (1998), the MPN method was applied to find out whether *C. tyrobutyricum* was the clostridium in milk or not. The results showed that there were low concentrations of Clostridia spp. in milk.

The advantage of the MPN method is that it can be used for solutions containing low concentrations of bacteria (Andersson & Christiansson, 1992). A disadvantage of the MPN- method is that it take longer time than the qPCR (approximately 3-7 days). Another disadvantage for this method is that it is not specific for *C. tyrobutyricum* (Cremonesi *et al*., 2012).

Plate count method

The plate count method is used to estimate number of different bacteria in milk and fodder (Andersen, 1951; Fu *et al*., 2006). Plate count is also a common method to estimate clostridia (Khamaiseh *et al*. 2012; Weimer & Stevenson, 2012; Rajagopalan & He, 2014).

The substrate for clostridia includes components such as nitrogen, amino acids, vitamins, carbon sources, sometimes components that react in contact with clostridia i.e. change colour and substrates that inhibit specifically unwanted microorganisms. A component that can react in presence with the clostridia is the redox-indicator neutral red. Neutral red changes to yellow when an acid-producing colony of clostridia grows on the medium (Jonsson, 1990). The colour change is due to the production of butyric acid by the clostridia when fermenting in the medium. Neutral red changes colour at acidic pH values (Kaufman & Weaver, 1959). D-Cycloserine works by inhibiting specifically unwanted microorganisms because it is an antibiotic, which inhibits the growth of *Bacillus spp*. (Jonsson, 1990). The inhibition is based on the inhibition of alanine racemase, an enzyme that catalyses the L-alanine formation in to D-alanine, an amino acid that is a very important component of the cell wall in *Bacillius* (Omotade *et al*., 2013).

Lactate dehydrogenase activity can be tested to verify clostridium fermenting lactic acid. Lactate is the only carbon source available for the clostridium in the test. In presence of lactate the reaction is catalysed by NADH-independent lactate dehydrogenase (iLDH) which means that hydrogen is released as well as NADH and pyruvate is formed. Furthermore, this fermentation produces hydrogen gas, carbon dioxide, acetic acid and butyrate (Zhu & Yang, 2004). Phenazin methosulfat acts as an electron carrier between iodonitro tetrazolium chloride and lactate dehydrogenase in this case. It has a yellow/green colour in the oxidized form and is colourless in reduced form (Worsfold *et al*., 1977). Tris-hydroxymethyl-amino-methane has a buffering capacity and makes the solution retain the pH-value (Sirieix *et al*., 1997).

The main advantage of the plate count method is that the method is relatively simple and requires no special or expensive equipment to perform the method and those colonies easily can be isolated directly from the plate. The disadvantages are that it is more time consuming than MPN (about 7 days) and that anaerobic incubators are needed.

Materials and Methods

Collection of samples

A sward containing 75% red clover and 25% grass was harvested on the 4th of September 2011 (third harvest) at Skara in the southwest of Sweden. The sward was mowed and wilted to 30-35% DM. The forage was chopped and baled with a stationary baler, (Compacter 2000, Orkel, Norway) using 8 layers of plastic film. The wilted and chopped forage was also packed in 1.7-l laboratory silos (Figure 5).

Figure 5. Laboratory silos.

Silage from the laboratory silos was used in this study. The wilted herbage contained on a DM basis: 16% crude protein, 10% ash, 10% WSC and 44% NDF. Following silage additives (Addcon Europe GmbH) wer added to the wilted forage at chopping; Kofasil LAC containing homofermentative LAB (*Lactobacillus plantarum* DSM 3676, DSM 3677) at an application rate of 1 $*10^5$ CFU/g forage, Kofasil S containing hetrofermntative *L. buchneri* (DSM 13573) $1*10^5$ CFU/g forage, Kofasil DUO containing a combination of Kofasil LAC and Kofasil S , at a dosage of $2*10^5$ CFU/g, and Kofasil COMBI a

combination of Kofasil LAC ($1*10^5$ CFU/g,) plus sodium benzoate, and potassium sorbate (270/g; Table 1).

The additive treated silages were compared to an untreated control. There were 3 replicates (silos) per treatment. The silos were stored at room temperature (20ºC) for 142 days. Once the silos had been emptied, the silages were stored in sterile bags at −20 °C until analysis of clostridia.

Analysis of the silage

The silage fermentation parameters were analyzed by the Central Laboratory, Humboldt University, Berlin, Germany. Lactic acid was analysed by HPLC (Weiss & Kaiser, 1995), volatile fatty acids and alcohols were analysed with gas chromatography (Weiss, 2001) and pH with an electrode. The water soluble carbohydrates (WSC) were analysed with the anthrone method (Lengerken & Zimmermann, 1991) and ammonia-nitrogen was determined calorimetrically with scalar CFSA based on the Berthelot reaction (Berhelot, 1859). Dry-matter (DM) concentration of the silages were corrected for losses of volatiles according to Weissbach and Strubelt (2008) (Table 1).

Table 1 Silage treatment, *C.tyrobutyricum* analysis and fermentation characteristics

Sample	Treatment	MPN-D [a]	MPN-S [b]	Plate [c]	Bacillus [d]	qPCR [e]	Dmcorr [f]	pH3days [g]	pHfinal [h]	LA [i]	AA [j]	PA [k]	BA [l]	Methanol	Ethanol	Propanol	Butanol	1,2-Propandiol	NH3NTOTN [m]	WSC [n]
Unit		log MPN/g	log MPN/g	log CFU/g	log CFU/g	log copies/g	%			% DM	% DM	% DM	% DM	% DM	% DM	% DM	% DM	% DM	% tot N	% DM
1	Kofasil S	4.0	4.5	4.7	5.0	5.8	29.5	4.9	4.9	3.1	4.9	0.9	0.7	0.3	0.7	0.9	0.000	0.2	15.1	0.3
2	Kofasil S	6.0	7.0	5.1	5.9	6.6	28.7	4.9	4.9	3.2	4.2	0.8	1.1	0.3	0.7	0.8	0.023	0.0	14.1	0.3
3	Kofasil S	6.0	7.0	5.0	6.7	6.1	29.6	4.9	4.9	3.0	4.9	1.0	0.9	0.3	0.7	0.9	0.022	0.0	13.3	0.3
4	Control	4.2	5.0	4.2	4.9	6.4	31.1	5.1	4.5	6.5	3.4	0.3	0.7	0.3	0.7	0.4	0.000	0.1	12.3	0.5
5	Control	5.2	5.4	3.0	5.6	5.9	27.4	5.2	4.6	8.1	4.0	0.4	0.9	0.4	0.8	0.6	0.000	0.1	13.9	0.5
6	Control	5.3	6.3	5.3	5.7	7.0	30.9	5.2	4.7	6.6	3.3	0.4	1.0	0.3	0.7	0.5	0.000	0.1	12.7	0.4
7	Kofasil Duo	3.5	2.6	4.7	4.1	3.4	36.1	4.4	4.3	9.6	2.3	0.0	0.0	0.3	0.3	0.0	0.000	0.3	8.7	0.7
8	Kofasil Duo	1.3	2.4	1.7	5.1	3.3	35.7	4.5	4.3	9.6	2.3	0.0	0.0	0.3	0.3	0.0	0.000	0.4	8.0	0.6
9	Kofasil Duo	1.3	2.0	2.2	4.2	3.3	35.6	4.6	4.3	9.7	2.3	0.0	0.0	0.3	0.3	0.0	0.000	0.4	8.4	0.6
10	Kofasil Lac	4.2	2.5	1.2	4.4	3.5	33.7	4.5	4.3	9.9	2.6	0.1	0.0	0.3	0.3	0.1	0.000	0.2	9.8	0.4
11	Kofasil Lac	5.5	3.5	2.7	4.8	2.7	33.2	4.4	4.3	9.9	2.6	0.1	0.0	0.3	0.3	0.1	0.000	0.2	9.4	0.4
12	Kofasil Lac	1.3	1.0	2.9	3.6	3.1	32.5	4.5	4.3	10.0	2.7	0.1	0.0	0.3	0.3	0.1	0.000	0.1	9.5	0.4
14	Kofasil Combi	4.2	2.5	1.2	4.9	3.3	35.2	4.5	4.2	11.3	2.0	0.0	0.0	0.3	0.2	0.0	0.000	0.1	8.6	0.8
15	Kofasil Combi	1.3	0.9	2.3	2.9	2.7	35.3	4.5	4.2	11.2	2.1	0.0	0.0	0.3	0.3	0.0	0.000	0.1	9.4	0.7

a) Most probable number method Germany.
b) Most probable number method Skara, Sweden
c) Plate method.
d) Bacillus on plate.
e) quantitative **P**olymerase **C**hain **R**eaction.
f) Corrected dry matter.
g) pH-value after 3 days in the silage.
h) final pH in the silage.
i) Lactic acid.
j) Acetic acid.
k) Propionic acid.
l) Butyric acid.
m) Total ammonium-N.
n) Water- soluble carbohydrate.

Silage sample preparation for all methods

Twenty grams of silage were weighed into a sterile bag and mixed with 180 g of 0.2% peptone and distilled water. The bag was sealed and kneaded by hand (instead of Stomacher) for 2 minutes. Thereafter, 10 ml of the liquid in the pouch were added to a sterile test tube. The test tube was placed in a water bath (80 °C) for 10 minutes to activate spores.

qPCR

Extraction

Two subsamples from each silage sample were taken and prepared the same way as the silage samples for the plate method and the MPN methods, but without the water bath step. From each sterile bag 1.5 ml of the silage solution were collected in to a tube. The tubes were centrifuged (10 min, 13000*g), to separate liquid phase from a resulting pellet. The pellet was then resuspended in 980 µl of Sodium Phosphate Buffer and 125 µl of MT Buffer (Fast DNA Spin Kit for Soil, MP Biomedicals) together with 22 µl of lysozyme (50 mg ml^{-1}, Roche Diagnostics Corporation, Indianapolis, IN, USA) to weaken cell walls. The tubes were gently vortexed and incubated for 1 hour at 37°C. The suspension was then added to a Lysing Matrix E tube containing 1.4 mm ceramic spheres, 0.1 mm silica spheres, and 4mm glass beads. The Lysing Matrix E tubes were vortexed. The tubes were then shaken 4 times for 50 s at a speed of 6.5 m/s using FastPrep instrument (MP Biomedicals) and cooled on ice for 2 min in between bead beatings. The samples were left for 30 min) to allow lysis to take place (RT). After that samples were centrifuged for 5.5 min (13000*g). About 1 ml of supernatant was transfered to the new 2 ml eppendorf tube with 250µl Binding Matrix (PPS) and inverted 10x by hand. After centrifugation for 5.5 min at 13000*g the supernatant was added to the 15 ml tube with 1 ml of Binding Matrix Suspension. The tubes were inverted by hand for 2 min to allow binding of DNA and left in rack for 3 min to allow settling of silica matrix. 600 µl of supernatant was discarded and the rest was resuspended by gentle vortexing. 600µl of suspension was added to SPINFilter and centrifuged for 1.5 min (13000*g), the catch tube was emptied. The remaining supernatant from the 15 ml tube was transferred to SPINFilter and treated in the same way. After that 500 µl SEWS-M (Salt/Ethanol Wash Solution) were added to the SPINFilter, centrifuged for 1.5 min (13000*g), the catch tube was emptied and centrifuged for 2 min (13000*g). The SPINFilter was placed in a clean Catch Tube and air dried for 5 min at RT. To elude the DNA from the SPINFilter, 100 µl of DNA - Elution - Solution – Ultra - Pure - Water (DES) was added and resuspended by carefully stirring with a pipette tip on the edge. The Catch Tube was then centrifuged for 1.5 min (13000*g) (modified method according to Bassi *et al*, 2013 and Wallenhammar *et al.*, 2012).

PCR-analysis

Primers' and probe's sequence as well as real-time qPCR conditions were the same as described by Bassia *et al.* (2013). The primers ptaF and ptaR (Table 2) were used to amplify a 150 bp fragment of phosphotransacetylase (pta) gene (accession number AY572855) and a probe ptaP with FAM as the 5' terminal reporter dye and TAMRA as 3' quencher was used to improve qPCR specifity. Real-Time quantitative PCR was performed using 7300 Real Time PCR Sysem (applied Biosystems) in a total volume of 25 µl. The qPCR reaction mixture included: 20 µl of Master Mix (1x TaqMan Universal PCR Master Mix No AmpErase UNG (Life Technologies), 0.4 µm ptaF, 0.4 µm ptaR, 0.1 µm ptaP) and

5 µl of DNA sample (diluted 5x with MQ H_2O) or 5 µl of standard DNA (plasmid with target gene diluted in a ten-fold scale up to 10^{10}). The thermal cycling conditions were as follows: pre-PCR denaturation for 10 min at 95°C , and then 45 cycles consisting of a 10 s at 95°C, 40 s at 60°C and 1 s at 72°C. All DNA samples were analysed in duplicates. The amount of bacterial DNA was quantified using a standard curve obtained for a 10-fold dilution series of the plasmid containing the *C. tyrobutyricum* target sequence (pta-gene) Auto baseline and auto threshold settings were used for results analysis. Data was exported to the template Excel file and based on the samples and standard Ct-values, the amount of gene copies per g of silage was calculated.

Table 2. The Probe, forward- and reverse primer used (Bassi *et al.*, 2013)

Primer/probe	**Sequence**
Primer forward (ptaF)	5'- AAG GGA AGT GCA CAA CAT GA -3'
Primer reverse (ptaR)	5'- ACT ACC AGG TGC TTT TAA ATT TGC -3'
Probe (ptaP)	5'- TGC TAA ACA AAT GCA ACC AGA -3'

MPN-S method

Silage sample preparation was done by dilution in test tubes in a 10-fold dilution series (between 10^{-1} and $10^{-6)}$ in triplicate for each dilution. Each test tube contained 9 ml of the Bryant Burkey broth (BBB) substrate BBB (containing; calcium lactate 5.0 g/liter; meat extract 7.5 g/liter; sodium acetate 5.0 g/liter; eptone from casein 15.0 g/liter; cysteine HCl 0.5 g/liter; resazurin 0.0025 g/liter; yeast extract 5.0 g/liter), a durham tube (to collect gas formation) and 1 ml of silage sample (Silage sample preparation for all methods). The samples were covered with 1 cm of 3% agar to create an anaerobe atmosphere. The test tubes were incubated at 37 °C for 7 days. Samples with a gas generated were considered positive for spores (Cerf and Bergere, 1968).

Calculation of probability

By using an MPN index table with 95% confidence intervals (FDA, 1978) the most probable *C. tyrobutyricum* content in the samples was estimated (Table 3).

Table 3. MPN table for a three-replicate design form (modify from FDA, 1978)

Positive tubes					Positive tubes				
0.1 (mL)	**0.01 (mL)**	**0.001 (mL)**	**MPN[a] (logMPN/mL)**	**95% Confidence range**	**0.1 (mL)**	**0.01 (mL)**	**0.001 (mL)**	**MPN[a] (logMPN/mL)**	**95% Confidence interval**
0	0	0	<3.0	0-9.5	2	2	0	21	4.5-42
0	0	1	3.0	0.15-9.6	2	2	1	28	8.7-94
0	1	0	3.0	0.15-11	2	2	2	35	8.7-94
0	1	1	6.1	1.2-18	2	3	0	29	8.7-94
0	2	0	6.2	1.2-18	2	3	1	36	8.7-94
0	3	0	9.4	3.6-38	3	0	0	23	4.6-94
1	0	0	3.6	0.17-18	3	0	1	38	8.7-110
1	0	1	7.2	1.3-18	3	0	2	64	17-180
1	0	2	11	3.6-38	3	1	0	43	9-180
1	1	0	7.4	**1.3-20**	3	1	1	75	17-200
1	1	1	11	3.6-38	3	1	2	120	37-420
1	2	0	11	3.6-42	3	1	3	160	40-420
1	2	1	15	4.5-42	3	2	0	93	18-420
1	3	0	16	4.5-42	3	2	1	150	37-420
2	0	0	9.2	1.4-38	3	2	2	210	40-430
2	0	1	14	3.6-42	3	2	3	290	90-1000
2	0	2	20	4.5-42	3	3	0	240	42-1000
2	1	0	15	3.7-42	3	3	1	460	90-2000
2	1	1	20	4.5-42	3	3	2	1100	180-4100
2	1	2	27	8.7-94	3	3	3	>1100	420-4000

a) Most probable number method

MPN-D

The German MPN method (MPN-D) was done at Landwirtschaftliche Kommunikations und Service mbH und Boden Futtermittellabor Mikrobiologisches Labor, FMUAA-159, LKS mbH, Lichtenwalde, Germany. Silage sample preparation was done as the "Silage sample preparation" but 40 g silage was used instead of 20 g and diluted 10 times with distillated water. A stomacher was used for 2 minutes.

A silage sample was diluted in a 10-fold dilution series (between 10^{-2} and 10^{-5}) and heated in 85°C for 5 minutes. After cooling, 100 µl triplicate of each dilution, 600 µl Lactat-Acetat-Agar contained: 30 ml sodiumlactate solution, 50% 10 g yeast extract, 8 g sodium acetate x 3H O,5 g Caseinpepton, 10 g Agar (heated to 50ºC) was applied in test tubes. The test tubes were incubated at 37 °C for 7 days. Samples with gas generated were considered positive for spores (Pahlow, 2003).

Plate method

Silage sample preparation was done as the "Silage sample preparation" described before (s. 23). For the plate method a Reinforced Clostridia (RCM-agar), consisting mainly of yeast extract, beef extract, peptone and agar, was used. These components provide the clostridia with nitrogen, amino acids, vitamins and carbon sources. The agars provide a semi-stable surface that allows spreading of the sample. Serial dilutions were made with 1 ml silage sample and 9 ml of 0.2% peptone fluid. 0.1 ml of the dilution was spread on two RCM agar plates for each dilution. The plates were placed in an anaerobic jar with a microbial anaerobe incubation kit (anaerobic cult) to get an anaerobic atmosphere and microbial

anaerobic test indicator (changes colour from blue to white in anaerobic environment). 35 ml of water were added to the anaerobic cult. The samples were incubated at 37°C for 7 days (Jonsson, 1990).

Calculation of C.tyrobutyricum

To determine amount of *C. tyrobutyricum* per gram of silage, the colonies on the plates were counted and following formula was used:

$$\frac{\Sigma Ci}{\Sigma Wi} = M$$

Where W stands for the dilution factor, C is the number of colonies on the corresponding dilution, i stand for the specific dilution and M stands for the clostridia per gram of silage (Niemelä, 1984).

Lactate dehydrogenase test (LDH)

Lactate dehydrogenase activity can be tested to verify clostridium fermenting lactic acid It is often used on lab (Zhu & Yang, 2004). Three colonies from each RCM plate were collected with a loop-needle and added to an eppendorf tube containing 0.5 ml of 0.1M DL-lactate at pH 5.5 and 0.3 ml of 1M tris hydroxymethyl-amino-methane at pH 8.5. The sample tubes were incubated in a water bath at 37°C for 5 min. Then 0.2 ml of the reagent solution containing 4 mM iodo- nitrotetrazoliumchloride and 1.6 mM phenazinmethosulfat were added to each tube. Tubes positive for fermenting lactic acid had a red coloured precipitation and tubes negative for fermenting lactic acid had light green precipitation (Jonsson, 1990). Microscopy was performed to verify presence of spores with a phase contrast microscope.

Statistical analyses

Relationships between clostridia spore counts from the traditional laboratory methods (the agar plate method and the MPN methods) and the qPCR method were investigated by linear regression analysis using PROC REG in SAS (ver. 9.4) , where regression models, *P*-value, R^2-value and mean square error (MSE) for the model were computed. Also, the least-square means of clostridia spore counts from the analytical methods were compared in the general linear model procedure (PROC GLM) of SAS (ver. 9.3). In this analysis, number of silage samples (n = 14) was used as replicates.

Stepwise regression on relationships between silage fermentation variables (DM, pH after three days of fermentation, pH final at opening of the silos after 143 days of storage, lactic acid, acetic acid, propionic acid, butyric acid, methanol, ethanol, propanol, butanol, 1,2-propandiol, 2,3-butandiol, ammonia-nitrogen (NH_3- N) and WSC) and clostridia spore counts from qPCR method, the MPN methods and the plate method, was analysed in PROC REG of SAS (ver. 9.3), where the fermentation variables were x-variables and the clostridia count from each method was the y-variable. As butyric acid has a strong relationship with clostridia activity, the stepwise regression analysis was done both with and without butyric acid in the set of x-variables. The x-variables resulting in a relationships with the clostridia spore counts for each analytical method with the highest R^2 value, where each x-variable has a *P*-value below 0.150 were selected in the stepwise

regression analysis. Results from the stepwise regression analysis are shown with *P*-values for each included x-variable as well as the mean square error (MSE) and R^2 value for the relationship. In addition, single relationships between silage fermentation products and the *Clostridium* content of the silage within each method was computed as linear regressions in Microsoft Excel (2016) with regression model and the R^2 value for the model.

Results

Comparisons of *C. tyrobutyricum* analysis methods

There was generally a larger variation between replicates within the same silage additive was treatment for the MPN-D, MPN-S and the Plate method compared to the qPCR method, especially for the samples with low clostridia spores (samples 7-14). There was also variation in the amount of clostridium spores between methods within the same sample. There is an effect of type of inoculant on the Clostridia spore count where the combination product of homo- and heterofermentative LAB and the products containing homofermentative LAB-n without and with the salts had less amounts of Clostridia than the untreated control and the silage treated with heterofermentative LAB (Figure 6).

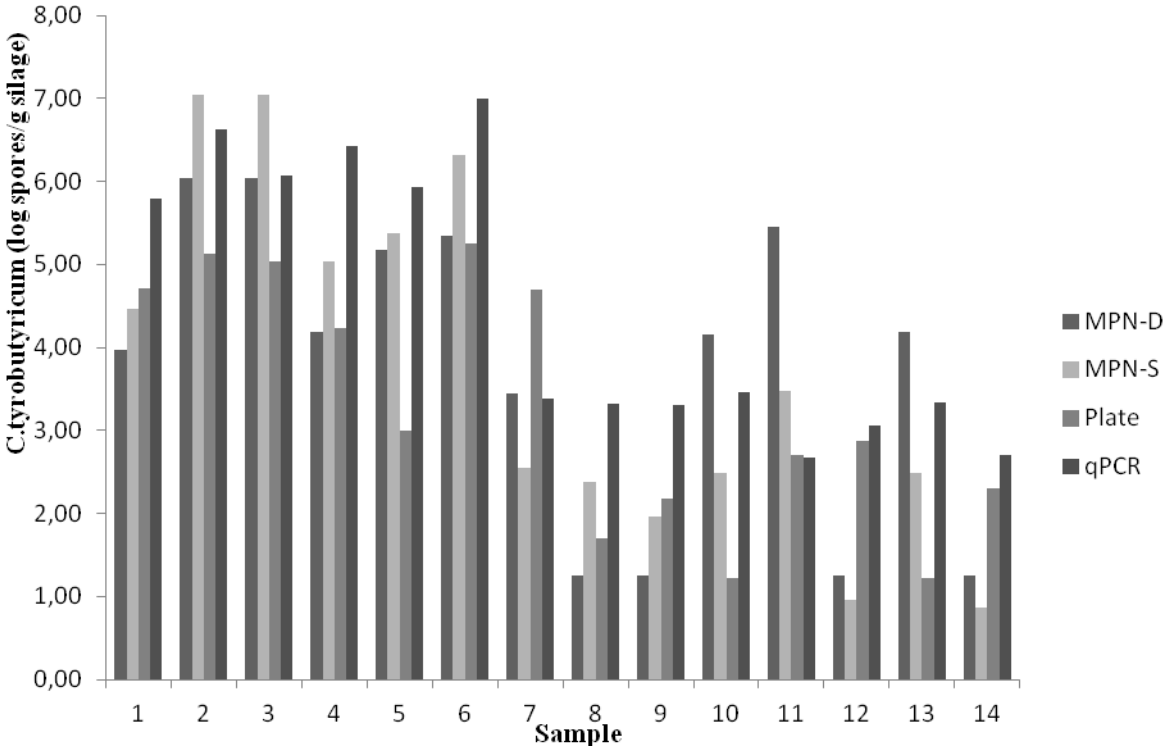

Figure 6. Total *Clostridium* in red clover/grass silage from each sample for all methods.

There were 3 samples (replicates) from each inoculant treatment and the untreated control, where the first 3 samples wear treated with Kofasil S (heterofermentative lactic acid bacteria (LAB) and the next 3 samples was untreated control samples. Samples 7, 8 and 9 was treated with Kofasil Duo (homo- and heterofermentative LAB), samples 10, 11 and 12 are treated with Kofasil Lac (homofermentative LAB) and the last 2 samples was treated with Kofasil Combi (homofermentative LAB plus potassium sorbate and sodium benzoate). For more details regarding the additive treatments, see Materials and Methods.

The relationship between the MPN-S and the qPCR method was strong ($R^2 = 0.83$) with a small deviation around the regression line (Figure 7). The MPN-D had a large deviation from the regression line, with a low correlation ($R^2 = 0.43$) between MPN-D and qPCR (Figure 8). Generally the qPCR method showed a higher value of *C. tyrobutyricum* in silage than the plate method (Figure 9). The plate method had some deviation from the

regression line in Clostridia count where the relationship between the plate method and the qPCR was moderate (R^2 = 0.57; Figure 9).

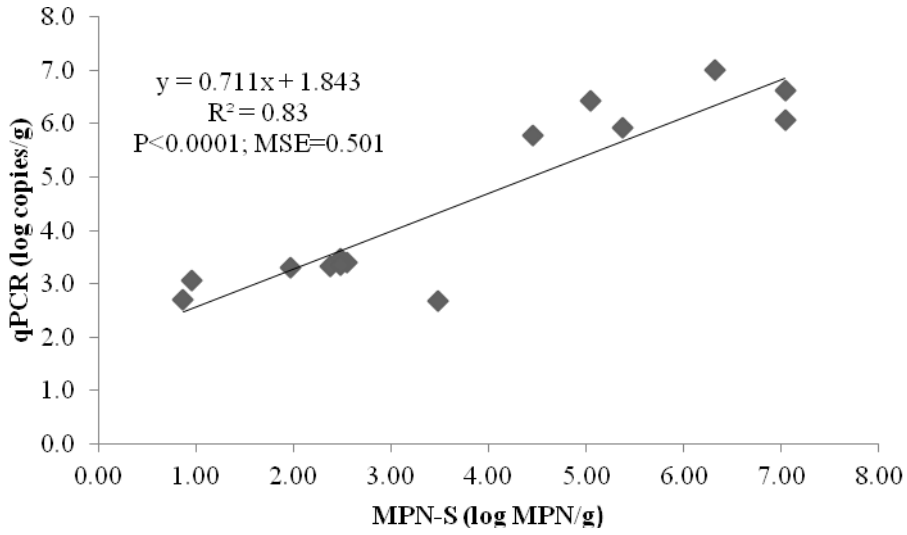

Figure 7. Relationship between total Clostridium in red clover/grass silage from the MPN-S method and the qPCR method.

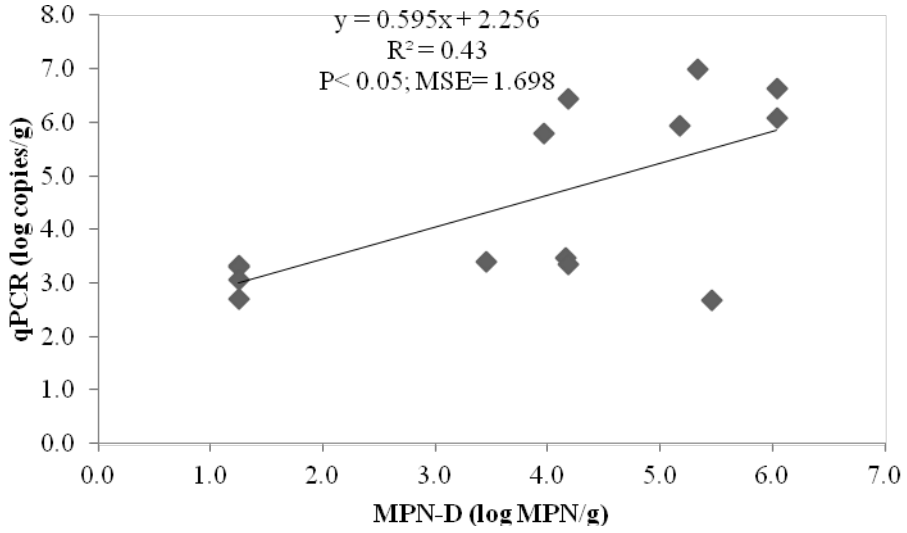

Figure 8. Relationship between total Clostridium in red clover/grass silage from the MPN-D method and the qPCR method.

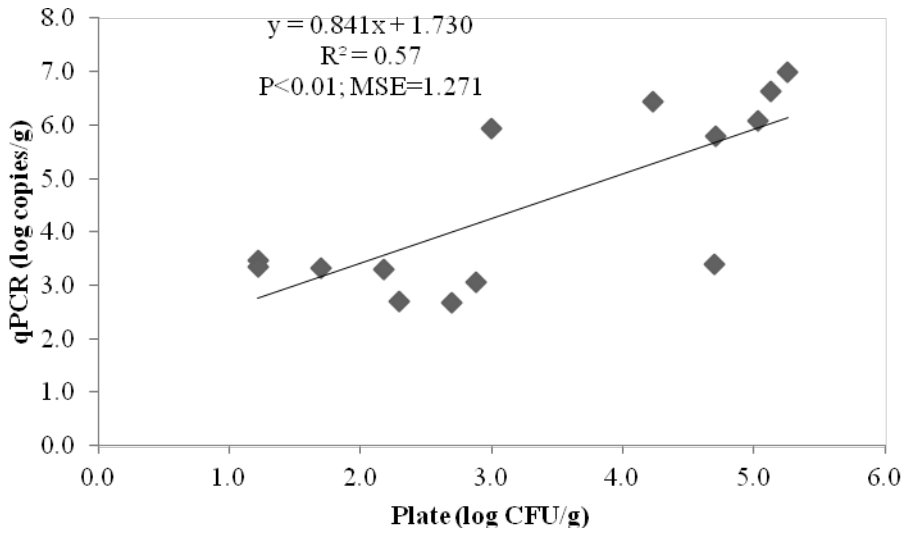

Figure 9. Relationship between total *C. tyrobutyricum* in red clover/grass silage from the plate method and the qPCR method.

Comparisons of the least-square (LS) means of clostridia spore count from the different methods did not show any significant differences between the methods (Table 4). The mean clostridium count in the silage was numerically but not significantly higher with the qPCR method compared to the plate-, MPN-D and MPN-S methods (Table 4).

Table 4. Mean count of clostridia spores detected by the qPCR (log copies/g)-, MPN-S (log MPN/g), MPN-D-(log MPN/g) and plate methods (log CFU/g). Least square (LS) means and standard error of the LS means (SEM), n = 14 silage samples.

Method	**qPCR**	**MPN-S**	**MPN-D**	**Plate**	**SEM**	***P* - value**
Clostridia	4.5	3.8	3.8	3.3	0.48	0.367

Relationships between *C. tyrobutyricum* count and fermentation characteristics of the silage for each method

qPCR

There were clear differences between the four *Clostridium* analysis methods when it comes to the relationships between *Clostridium* counts and silage fermentation parameters (Tables 5 and 6).

Variations in counts of *C. tyrobutyricum* by the qPCR method could to 98% (R^2 = 0.98) be explained by variations in silage pH after 3 days of fermentation (Figure 10) and variations in contents of lactic acid (Figure 11), acetic acid (Figure 12) and 1, 2–propanediol (Figure 13) of the silage with only minor deviations from the regression line (MSE = 0.0571; Table 5). When butyric acid was excluded from the regression analysis, variations in ethanol concentrations of the silage explained 92% of the variation in *C. tyrobutyricum* counts when the qPCR method was used (Table 6; Figure 14).

Results from the stepwise regression analysis did not include the ammonia, but simple linear regressions of the *Clostridium* and ammonia showed that the ammonia was increasing with increasing *Clostridium* in the silage (Figure 15; Table 7). The butyric acid was also not selected by SAS as a parameter affecting the count of *C. tyrobutyricum* in the silage when using the qPCR (Table 5) method although and table 17 shows a clear relationship between butyric acid content and the *C. tyrobutyricum* count (Figure 16).

Table 5. Relationships between fermentation characteristics and *Clostridium* count of the silage for each analysis method including butyric acid selected by the stepwise regression analysis. MSE = mean square error, n = 14 silage samples.

Method	**Fermentation parameter**	***P*- value**	**MSE**	**R^2**
qPCR	pH 3 days	< 0.0001		
	Lactic acid	< 0.001		
	Acetic acid	< 0.05		
	1, 2-Propanediol	0.107	0.0571	0.98
MPN-S	Butyric acid	< 0.0001		
	Butanol	0.113	0.517	0.90
MPN-D	Butyric acid	< 0.01	1.875	0.48
Plate	Lactic acid	< 0.001	0.868	0.64

Table 6. Relationship between fermentation characteristics and *Clostridium* count of the silage for each analysis method excluding butyric acid selected by the stepwise regression analysis. MSE = mean square error, n = 14 silage samples.

Method	**Fermentation parameter**	***P*-value**	**MSE**	**R^2**
qPCR	Ethanol	< 0.001	0.211	0.92
MPN-S	pH-final	< 0.001		
	Propionic acid	< 0.05	0.684	0.87
MPN-D	DM	< 0.01	1.956	0.46
Plate	Lactic acid	< 0.001	0.868	0.64

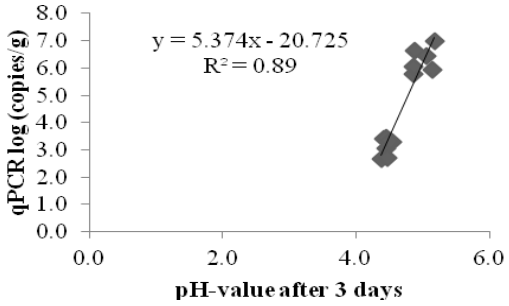

Figure 10.Relationship between silage pH after 3 days of fermentation and *C. tyrobutyricum* count by the qPCR method.

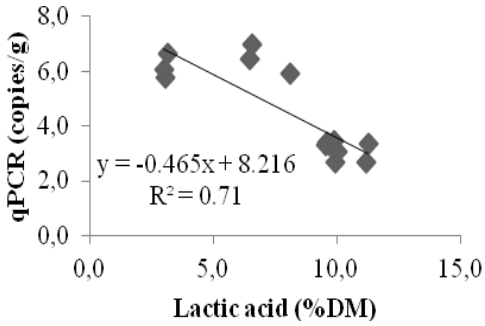

Figure 11. Relationship between silage lactic acid content and *C. tyrobutyricum* count by the qPCR method.

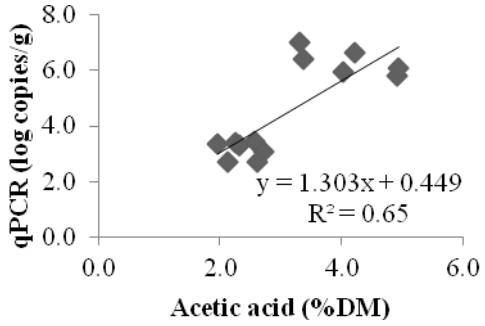

Figure 12. Relationship between silage acetic acid content and *C. tyrobutyricum* count by the qPCR method.

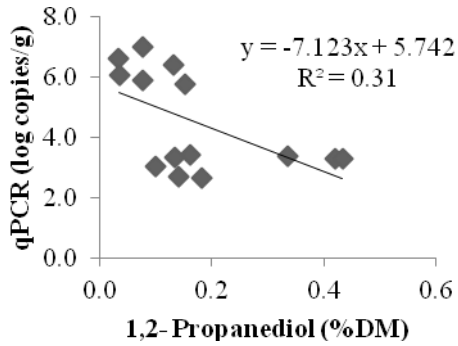

Figure 13. Relationship between silage 1.2-propanediol content and *C. tyrobutyricum* count by the qPCR method.

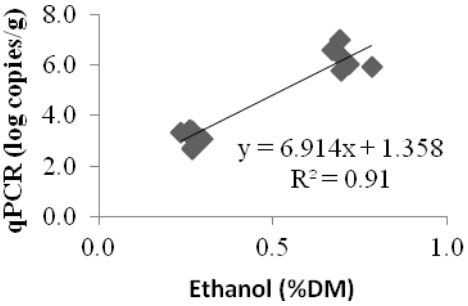

Figure 14. Relationship between silage ethanol content and *C. tyrobutyricum* count by the qPCR method.

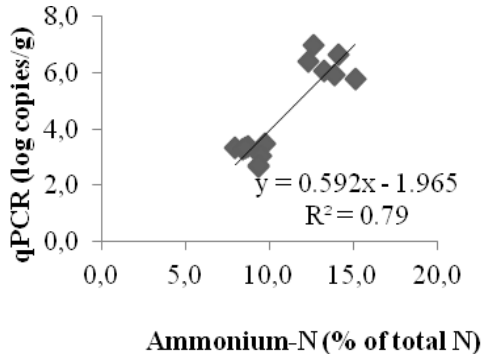

Figure 15. Relationship between silage ammonia-N content and *C. tyrobutyricum* count by the qPCR method.

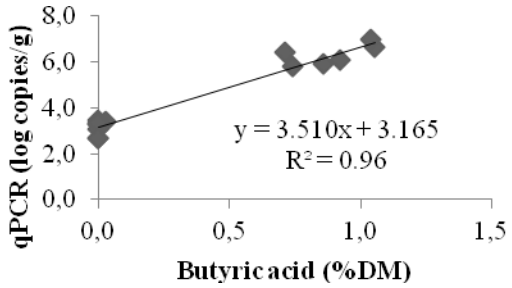

Figure 16. Relationship between silage butyric acid content and *C. tyrobutyricum* count by the qPCR method.

Table 7. Relationships between *clostridium* count by different methods and fermentations parameters in silage (all variables in % of DM except NH_3-N, which is in % of total N).

	Method							
	qPCR [a]		MPN-S [b]		MPN-D [c]		Plate [d]	
Variable	Regression	R^2	Regression	R^2	Regression	R^2	Regression	R^2
Dmcorr [e]	y = -0.4685x + 19.713	0.69	y = -0.5873x + 22.806	0.66	y = -0.4242x + 17.551	0.46	y = -0.3022x + 13.11	0.35
WSC [f]	y = -6.1229x + 7.5807	0.35	y = -8.2644x + 7.895	0.39	y = -5.8041x + 6.6993	0.26	y = -4.56x + 5.5933	0.24
Lactic acid	y = -0.4651x + 8.2162	0.71	y = -0.6008x + 8.538	0.72	y = -0.364x + 6.688	0.36	y = -0.3994x + 6.4896	0.64
Acetic acid	y = 1.3026x + 0.4492	0.65	y = 1.6823x - 1.4942	0.66	y = 1.0966x + 0.3697	0.38	y = 1.0234x + 0.1162	0.49
Propionic acid	y = 3.615x + 3.4747	0.6	y = 4.809x + 2.373	0.65	y = 3.1659x + 2.8816	0.38	y = 3.0125x + 2.4439	0.51
Butyric acid	y = 3.5097x + 3.1652	0.96	y = 4.3084x + 2.0991	0.88	y = 2.5227x + 2.942	0.46	y = 2.523x + 2.340	0.61
Ethanol	y = 6.914x + 1.358	0.91	y = 8.1544x + 0.0326	0.77	y = 5.0976x + 1.4641	0.41	y = 4.7643x + 1.1344	0.53
NH_3-N [g]	y = 0.5915x - 1.9651	0.79	y = 0.704x - 3.959	0.68	y = 0.4813x - 1.4811	0.43	y = 0.4266x - 1.3643	0.51
pH 3 days [h]	y = 5.3744x - 20.725	0.89	y = 6.7263x - 26.253	0.79	y = 3.4161x - 12.253	0.3	y = 3.1539x - 11.503	0.38
pH final [i]	y = 5.1701x - 18.552	0.77	y = 6.726x - 26.253	0.79	y = 4.312x - 15.446	0.44	y = 4.2131x - 15.486	0.63

a) quantitative **P**olymerase **C**hain **R**eaction.
b) Most probable number method Sweden.
c) Most probable number method Germany.
d) Plate count method.
e) Corrected dry matter.
f) Water soluble carbohydrate.
g) Total ammonium-N.
h) pH-value after three days.
i) Final pH-value.

MPN-S

When using the MPN-S method, variations in butyric acid (Figure 17) and buthanol (Figure 18) concentrations of the silage explained 90% (R^2 = 0.90) of the variations in the *Clostridium* counts (Table 4). When butyric acid was excluded from the analysis, the final pH (Figure 19) and contents of propionic acid (Figure 20) of the silage were chosen by the regression to explain 87% (R^2 = 0.87) of the variation in spore counts of the silage (Table 6).

The stepwise regression analysis did not select the ammonia and lactic acid when using the MPN-S method. Simple linear regressions of the MPN-S for spore counts content and ammonia-N showed that the ammonia-N was increasing with increasing spore counts in the silage (Figure 21; Table 7). The lactic acid was also not selected in the stepwise regression

analysis but simple regressions showed that presence of spore count affected the lactic acid content of the silage (Figure 22; Table 7).

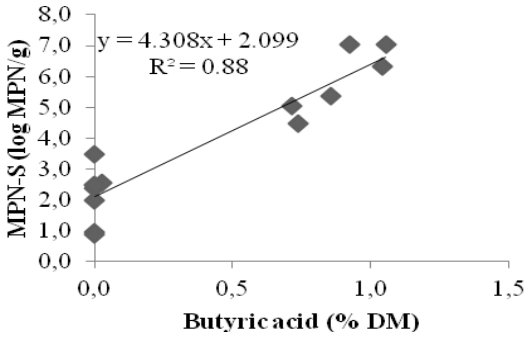

Figure 17. Relationship between silage butyric acid content and spore counts count by the MPN-S method.

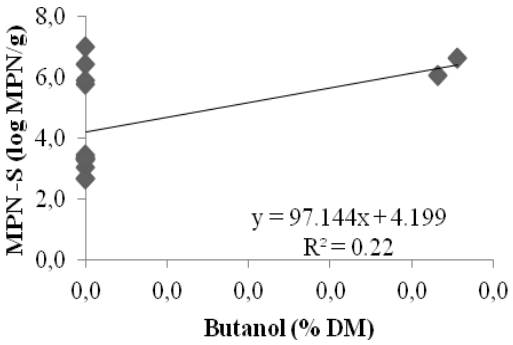

Figure 18. Relationship between silage butanol content and spore counts count by the MPN-S method.

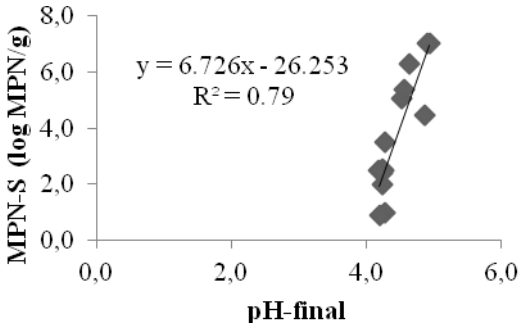

Figure 19. Relationship between silage pH final of fermentation and spore counts by the MPN-S method.

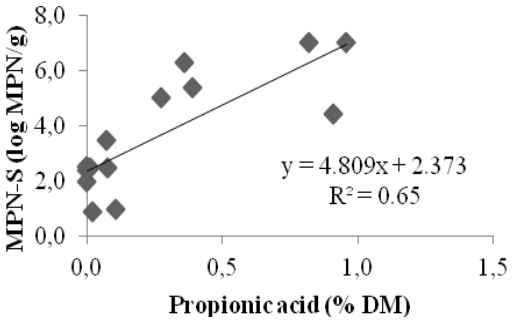

Figure 20 Relationship between silage propionic acid content and spore count by the MPN-S method.

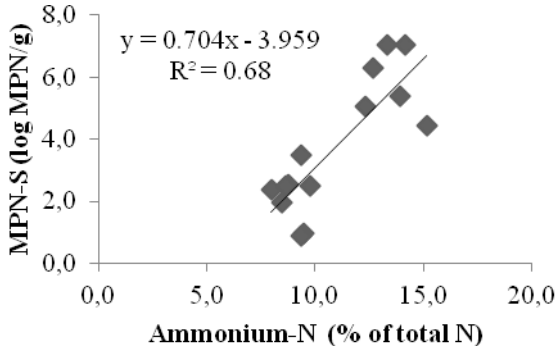

Figure 21. Relationship between silage ammonium-N content and spore count by the MPN-S method.

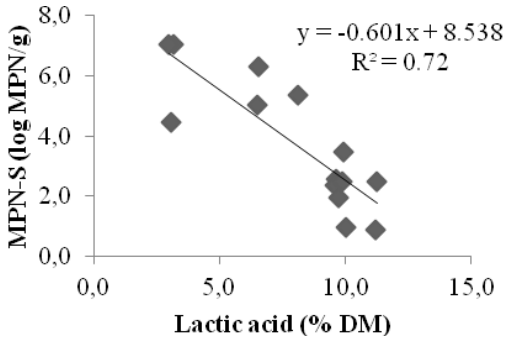

Figure 22. Relationship between silage lactic acid content and spore count by the MPN-S method.

MPN-D

When using the MPN-D method, variations in spore counts could to 48% (R^2 = 0.48) be explained by the variation in butyric acid (Table 5; Figure 23) with large deviations from the regression line (MSE = 1.874; Table 5). When the butyric acid was excluded from the analysis, the DM content of the silage explained 46% (R^2 = 0.46) of the variation in *clostridium* in the silage (Table 6; Figure 24).

The stepwise regression analysis did not select the ammonia-N and lactic acid contents when using the MPN-D method. Simple linear regressions of the MPN-D for *Clostridium* spore count and ammonia-N showed that the ammonia-N was increasing with increasing spore content in the silage (Figure 25; Table 7). Also, simple regressions showed that *Clostridium* spore count affects the lactic acid content of the silage (Figure 26; Table 7).

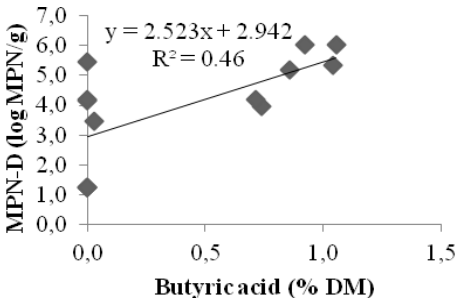

Figure 23. Relationship between silage butyric acid content and spore count by the MPN-D method.

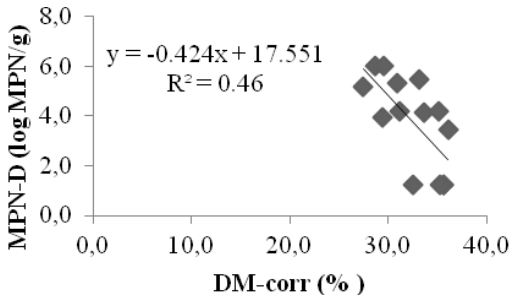

Figure 24. Relationship between silage dry matter and spore count by the MPN-D method.

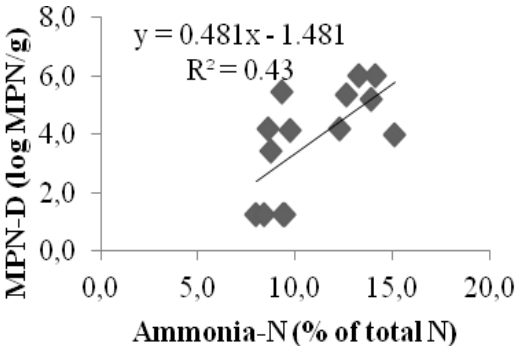

Figure 25. Relationship between silage ammonia-N and spore counts by the MPN-D method.

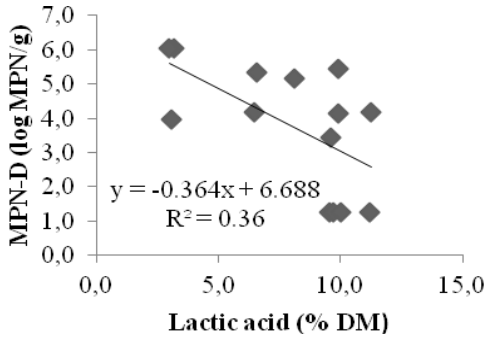

Figure 26. Relationship between silage dry matter content and spore count by the MPN-D method.

Plate

Variations in counts of *C. tyrobutyricum* by the plate method could to 64% (R^2 = 0.64) be explained by variations in lactic acid content of the silage. The deviation was relatively large from the regression line (MSE = 0.868; Table 5; Figure 27). When the butyric acid was excluded from the stepwise regression analysis the same results for lactic acid was given (Table 6).

The regression analysis did not include the ammonia-N and butyric acid contents when using the plate method. Simple linear regressions between *C. tyrobutyricum* of the plate method and ammonia-N showed that the ammonia increased with increasing *C. tyrobutyricum* in the silage (Figure 28; Table 7). The butyric acid was also not selected in the stepwise regression analysis but simple regressions showed that *C. tyrobutyricum* affects the butyric acid content of the silage (Figure 29; Table 7).

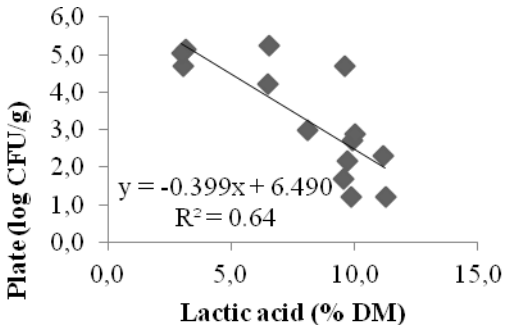

Figure 27. Relationship between silage lactic acid content and *C. tyrobutyricum* count by the plate method.

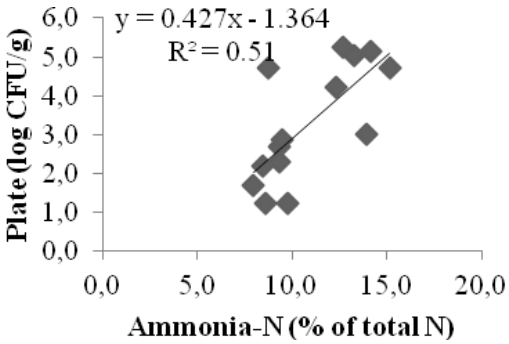

Figure 28. Relationship between silage ammonia-N content and *C. tyrobutyricum* count by the plate method.

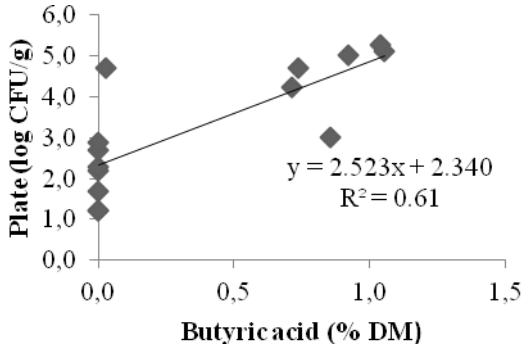

Figure 29. Relationship between silage butyric acid content and *C. tyrobutyricum* count by the plate method.

Discussion

Comparisons of C. tyrobutyricum analysis methods

MPN-S and qPCR

In this study the results showed that the relationship between the MPN-S- and qPCR methods was strong (R^2 = 0.83) with a small deviation around the regression line (Figure 7). This means that the variation in the MPN-S method could explain 83% of the total variation in *C. tyrobutyricum* content of clover-grass silage analyzed by the qPCR method. MPN is not a specific method for *C. tyrobutyricum* as qPCR. To benefit *C.tyrobutyricum*, BBB substrate (containing calcium lactate 5.0 g/liter; meat extract 7.5 g/liter; sodium

acetate 5.0 g/liter; eptone from casein 15.0 g/liter; cysteine HCl 0.5 g/liter; resazurin 0.0025 g/liter; yeast extract 5.0 g/liter) is used due to enable *C. tyrobutyricum* to produce gas in an anaerobic environment with access to lactate and acetate in the substrate (Cocolin *et al*. 2004). Therefore using only the MPN method to identify *C. tyrobutyricum* in silage does not suffice (Cremonesi *et al*., 2012). Another bacterium could affect the MPN method results. An example of bacteria that can affect the MPN results is *Bacillus* since *Bacillus* can ferment organic acids in silage, such as lactic acid and could therefore give false positive results in the MPN method (Cato *et al*., 1986; Andersson *et al*., 2008; Driehuis *et al*., 2009). In this study it was observed that *Bacillus* was growing on all plates and therefore it was concluded that *Bacillus* was present in the silage and may have affected the MPN results. It was no statistical difference between the MPN-S method and qPCR method (log 3.8 cfu/g vs. log 4.5 copies/g). Therefore, he MPN-S method could be used for estimating the *Clostridium* spore content in clover-grass silage.

MPN-D and qPCR

In this study it was a low correlation (R^2 = 0.43) between MPN-D and qPCR (Figure 8). The MPN-D is just as MPN-S not a specific method for *C. tyrobutyricum* as qPCR. Therefore if only using the MPN method, *C. tyrobutyricum* cannot be identified (Cremonesi *et al*., 2012). The low correlation between MPN-D and qPCR can depend on that it is not a specific method and for example the *Bacillus* could have affected the results. *Bacillus* can give false positive results since *Bacillus* can ferment lactic acid as *C. tyrobutyricum* (Andersson *et al*., 2008). Another explanation could be that proteolytic clostridia was fermenting or other spore forming bacteria was growing due to the method is unspecific (Pahlow *et al*. 2003; Cato *et al*., 1986). To benefit *C.tyrobutyricum*, an agar substrate containing lactate and acetate was used to suite *C.tyrobutyricum*. The different substrates used and the different laboratories used seem to affect the difference between the MPN-S and MPN-D results

Plate and qPCR

The correlation between the plate method and qPCR was moderate (R^2 = 0.57; Figure 9). The plate method is common to use for estimating clostridia (Khamaiseh *et al*. 2012; Weimer & Stevenson, 2012; Rajagopalan & He, 2014). The substrate, which is specific for clostridia include components, such as nitrogen, amino acids, vitamins, carbon sources, and components that react in contact with growing clostridia i.e. change colour and substrates that inhibit specifically unwanted microorganisms (Jonsson, 1990). The method had a lower correlation with qPCR (R = 0.57) than MPN-S and qPCR (R^2=0.87) and a low mean count compared to the qPCR method (3.3 cfu/g vs. 4.5 copies/g). The result shows that the plate method detects clostridia less frequent than the qPCR method. The plate method could be used to estimate the *C. tyrobutyricum* amount in silage but the MPN-S method seems to be a better alternative due to higher correlation (R^2=0.87) when it comes to analyze Clostridia spores in clover-grass silage. One advantage with plate count is that it enables easy isolation of strains for further identification.

Relationships between Clostridium count and fermentation characteristics of the clover-grass silage for each method

qPCR

In this study variations in copies of *C. tyrobutyricum* by the qPCR method could to 98% (R^2 = 0.98) be explained by variations in silage pH after 3 days of fermentation (Figure 8), lactic acid (Figure 11), acetic acid (Figure 12) and 1, 2–propanediol (Figure 13). The regressions indicates that the pH-value and the acetic acid content will increase while the concentrations of lactic acid and 1,2– propanediol will decrease with increasing content of *C. tyrobutyricum*. Studies by Van Beynum & Pette (1935) showed that the pH-value will increase in silage contaminated with *C. tyrobutyricum. The C. tyrobutyricum* ferment the lactic acid in to weaker acids butyric acid and acetic acid (Bryant, Burkey, 1956). The pH will therefore increase and the lactic acid decrease in silage containing high amounts of *C. tyrobutyricum* (Kung & Shaver, 2000). There is also a further explanation to the increased pH-value and that is the interaction between *C. tyrobutyricum* and yeast (Jonsson, 1990). In a study by Jonsson and Pahlow (1984) they observed that yeast can respire by use of lactic acid, which also lead to an increased pH-value in the silage. Acetic acid is a byproduct from the *C. tyrobutyricum* fermentation in silage with a pH-value about 5 (Zhu & Yang, 2004). In the stepwise regression the correlation to 1, 2–propanediol was selected. The selection could be explained by *L. diolivorans. L. diolivorans* interacts with *L. buchneri*, fermenting lactic acid, in to 1,2-propandiol and acetic acid. *L. diolivorans* will use the 1,2-propanediol to produce propionic acid and n-propanol (Oude Elferink *et al.*, 1997).

When butyric acid was excluded from the regression analysis, variations in ethanol concentrations of the silage explained 92% of the variation in *C. tyrobutyricum* copies when the qPCR method was used (Table 6; Figure 12). The regression indicates that the amount of ethanol increased with increasing amount of *C. tyrobutyricum* in silage. This correlation also depends on the interaction between yeast and *C. tyrobutyricum* in silage, as ethanol is produced when yeast respires (Jonsson, 1990). When yeast consumes lactic acid and glucose it mainly produces ethanol, propanol, butanol and pentanol (Kyuei *et al*. 1977; Middelhoven & van Baalen, 1988; Jonsson, 1991; Vissers *et al.* 2007; Tabacco *et al*. 2009). Ammonia-N and butyric acid were not selected from the regression analysis. Simple linear regressions of the *C. tyrobutyricum* and ammonium showed a high correlation with *C. tyrobutyricum* in silage (Figure 15; Table 7). This could be explained by growth of proteolytic clostridia or other ammonia-N producing bacteria, i.e. enterobacteria in the silage (Rooke & Hatfield, 2003; Zhu & Yang, 2004).

The high correlation (R^2=0.96) between *C. tyrobutyricum* by the qPCR method and butyric acid (Table 7) shows that the method is well adapted to use for quantification of *C. tyrobutyricum* in clover-grass silage. Butyric acid is produced by the *C. tyrobutyricum,* when fermenting glucose and lactic acid in silage (Van Beynum & Pette, 1935; Seglar, 2003; Carvalho *et al*., 2014). The result indicates that this qPCR method for *C. tyrobutyricum* in silage could be a good method to use and should be interesting to investigate further, which also Cremonesi *et al*., 2012; Bassi *et al*., 2013 and König *et al*. (2017) concluded.

MPN-S

The results showed that with the MPN-S method, variations in butyric acid (Figure 17) and butanol (Figure 18) concentrations of the silage explained 90% (R^2 = 0.90) of the variations in the Clostridia spore count (Table 5). Butyric acid is produced by the *C. tyrobutyricum* when fermenting in the silage (Van Beynum & Pette, 1935; Seglar, 2003; Carvalho *et al*., 2014). The amount of butyric acid will increase with the amount of *C. tyrobutyricum* in the silage (Seglar, 2003). Butanol is mainly produced by yeast when fermenting WSC in the silage (Kyuei *et al*. 1977). Yeast and *C. tyrobutyricum* interact with each other. Lactate-assimilating yeast consumes the lactic acid and produce water and carbon dioxide creating anaerobic atmosphere for the clostridia to grow in the silage (Beynum & Pette, 1935; Jonsson, 1989; Jonsson, 1991; Vissers *et al.* 2007; Tabacco *et al*. 2009). When the atmosphere in the silage is anaerobic the yeast starts fermenting WSC and produce alcohols (Pahlow *et al*., 2003).

When butyric acid was excluded from the analysis, the final pH (Figure 19) and contents of propionic acid (Figure 20) could explain 87% (R^2 = 0.87) of the variation in spore counts of the silage (Table 6). The final pH will increase in silage contaminated with *C. tyrobutyricum (*Van Beynum & Pette, 1935). The *C. tyrobutyricum* will consume the lactic acid, which is the main acid to hold the low pH in the silage (Kung & Shaver, 2000). Simple regressions between lactic acid and spore amount showed that increased spore count decreased the lactic acid content of the silage (Figure 22; Table 6). The *C. tyrobutyricum* is a saccharolytic bacterium and therefore consumes the lactic acid in the silage (Kung & Shaver, 2000; Rooke & Hatfield, 2003; Zhu & Yang, 2004). When consumed the lactic acid will produce weaker acid, butyric acid and acetic acid resulting in decrease lactic acid and increase in pH in the silage (Van Beynum & Pette, 1935). Also the *Bacillus* can consume the lactic acid and produce acetic acid, bytyric acid lactic acid, ethanol, glycerol or 2.3 butanediol (Claus & Berkeley, 1986), making the final pH increase in the silage (Driehuis *et al*., 2009). The *Bacillus* also compete with the lactic acid bacteria about the carbon recourses, which can inhibit the lactic acid bacteria resulting in the increase of pH (Woolford, 1977).

Propionic acid is produced from proteolytic clostridia in silage (Schwartz & Schäfer, 1973: Pahlow, 2003). This result seems to indicate that when *C. tyrobutyricum* grows in the silage the proteolytic clostridium also grows in the silage. Simple linear regressions of the MPN-S for Clostridia spore count and ammonia-N showed that the ammonia-N was increasing with increasing Clostridia spore count in the silage (Figure 25; Table 6). This could be explained by proteolytic clostridia growing or other ammonia-N producing bacteria for example entrobacteria (Rooke & Hatfield, 2003; Zhu & Yang, 2004).

The MPN method is not specific for *C.tyrobutyricum,* which can make false positive indications (Andersson *et al*., 2008). In this study it was observed that *Bacillus* was growing on all plates and therefore it was concluded that *Bacillus* was present in the silage and may have affected the MPN-S results. The *Bacillus* can ferment organic acids in the silage, such as lactic acid and could, therefore, result in false positive results with the MPN-S method (Cato *et al*., 1986; Andersson *et al*., 2008; Driehuis *et al*., 2009).

MPN-D

When using the MPN-D method in the study, variations in clostridia spore count could to 48% (R^2 = 0.48) be explained by the variation in butyric acid (Table 5; Figure 23). This correlation between Clostridia spore count and butyric acid is considered moderate, as it is well known that butyric acid are produced by the *C. tyrobutyricum* when present in the silage (Van Beynum & Pette, 1935; Seglar, 2003; Carvalho *et al*., 2014).

When butyric acid was excluded from the analysis, the DM content of the silage explained 46% (R^2 = 0.46) of the variation in *Clostridia* spore count in the silage (Table 6; Figure 24). The pH-value will increase in silage contaminated with Clostridia *(*Van Beynum & Pette, 1935). The *C. tyrobutyricum* will consume the lactic acid, which results in increase pH in the silage (Kung & Shaver, 2000). Jonsson (1990) also observed a higher pH-value on the surface of the silage bale compared to deeper inside the silage due to interaction between yeast and *C.tyrobutyricum*. *Bacillus* also often grows in silage containing clostridia and can be a contributing factor in increasing pH in silage. The *Bacillus* can compete with the lactic acid bacteria about the carbon recourses (Woolford, 1977).The *Bacillus* can also consume the lactic acid in to weaker acids (acetic acid, butyric acid lactic acid, ethanol, glycerol or 2.3 butanediol: Claus & Berkeley, 1986), making the pH increase in the silage (Driehuis *et al*., 2009).

The stepwise regression analysis did not select the lactic acid and ammonia-N contents when using the MPN-D method. Simple linear regressions of the MPN-D for spore content and lactic acid and ammonia-N showed correlations (Figure 25, 26; Table 7). It was unexpected that the stepwise regression analysis did not select the lactic acid since it is well known that *C. tyrobutyricum* ferment lactic acid (Henderson & McDonald, 1975; Kung & Shaver, 2000). One explanation could be that proteolytic clostridia were fermenting or other spore forming bacteria were growing as the method is not specific for *C. tyrobutyricum* (Cato *et al*., 1986; Pahlow *et al*. 2003). Simple linear regressions of the MPN-D for spore content and ammonia-N showed that the ammonia-N increased with increased amounts of spores. This could be explained by growth of proteolytic clostridia or other ammonia-N producing bacteria, i.e. entrobacteria (Rooke & Hatfield, 2003; Zhu & Yang, 2004).

As the MPN method is not specific for *C.tyrobutyricum*, the method can make false positive indications for example *Bacillus* (Cato *et al*., 1986; Andersson *et al*., 2008; Driehuis *et al*., 2009). Bacillus was observed in the silage and therefore it could affect the MPN-D results.

Plate count method on RCM-agar

In this study variations in counts of *C. tyrobutyricum* by the plate method could be explained to 64% (R^2 = 0.64) by the lactic acid concentration in the silage both with and without butyric acid in the stepwise regression analysis (Tables 5 and 6). The lactic acid concentration in the silage will decrease when the amount of clostridia increase in the silage (Figure 27). As mentioned earlier *C. tyrobutyricum* consumes the lactic acid in the silage (Kung & Shaver, 2000; Rooke & Hatfield, 2003; Zhu & Yang, 2004). Therefore this correlation (R^2 = 0.64) can be considered as good but the plate method is not as specific for *C. tyrobutyricum* count as the qPCR method (Van Beynum & Pette, 1935; McDonald, 1982).

The butyric acid was not selected in the stepwise regression analysis by the plate method. Simple regressions showed that *C. tyrobutyricum* increased the concentration of butyric acid (R^2=0.61) of the silage (Figure 29; Table 7). This correlation between *C. tyrobutyricum* and butyric acid is considered moderate, due to it is well known that butyric acid is produced by the *C. tyrobutyricum* when present in the silage (Van Beynum & Pette, 1935; Seglar, 2003; Carvalho *et al*., 2014).

The stepwise regression analysis did not include the ammonia-N contents when using the plate method. Simple linear regressions of the plate method and ammonia-N showed that the ammonia increased with increased *C. tyrobutyricum* in the silage (Figure 28; Table 7). The correlation of the plate method and ammonia-N was low (R^2=0.51) which can be explained by the saccharolytic activity of *C. tyrobutyricum* and no proteolytic activity (Pahlow *et al*. 2003; Rooke & Hatfield, 2003; Zhu & Yang, 2004).

Conclusions

There were no significant differences in Clostridia spore counts of red clover-grass silage between the qPCR, MPN-D, MPN-S and the plate methods when comparing the LS means of the clostridia counts from the different methods. However, when relating the clostridia spore count estimates in the silage analysed by the traditional MPN and plate count methods to the *C. tyrobutyricum* copies in the silage analysed by the qPCR method, the relations were significant. The high specificity for the qPCR method decreases the risk of counting other lactate degraders than *C. tyrobutyricum* in the silage. The simple linear regressions showed that the MPN-S and the plate method had moderate to strong correlations with the qPCR method, which indicates their reliability for estimating the *Clostridium* spore content in clover-grass silage. The MPN-D showed low correlations with the qPCR. The qPCR method showed very high correlation with the fermentation parameters compared to the MPN-D and the plate count methods. Therefore, the qPCR method for *C. tyrobutyricum* in silage is a relevant method to use and should be investigated further. Also, the MPN-S method showed promising relationships with the fermentation products in the silage. As the results are based on only 14 samples of red clover-grass silage harvested from one field, the variation in fermentation characteristics and clostridia spore count is caused by the different actions of the homo- and heterofermentative LAB occurring naturally in the forage and used in the silage additives applied to the herbage at ensiling. This initial small dataset needs to be extended with samples from different environments to make the relationships more robust between the MPN and plate count methods with the qPCR method. A large data set needs to be created and tested for correlations before predictions of the clostridia counts can be made from silage fermentation end products.

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