Doctoral Programme in Sustainable Use of Renewable Natural Resources
Doctoral School in Environmental, Food and Biological Sciences
University of Helsinki
Finland

EFFECTS OF ENSILABILITY TRAITS OF FORAGE LEGUMES AND ADDITIVES ON SILAGE QUALITY ASSESSED BY FERMENTATION PATTERN AND qPCR QUANTIFICATION OF CLOSTRIDIA

Walter König

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in lecture room Porthania P673, Yliopistonkatu 3, Helsinki, on 25 May 2020, at 12 o'clock.

Helsinki 2020

Supervisor: **Docent Seija Jaakkola**
Department of Agricultural Sciences
University of Helsinki, Finland

Pre-examiners: **Dr. Katrin Gerlach**
Institute for Animal Sciences
University of Bonn, Germany

Dr. Xusheng Guo
Probiotics and Biofeed Research
Lanzhou University, China

Opponent: **Associate Professor Rolf Spörndly**
Department of Animal Nutrition and Management
Swedish University of Agricultural Sciences, Sweden

Custos: **Professor Aila Vanhatalo**
Department of Agricultural Sciences
University of Helsinki, Finland

Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentariae, Biologicae, Publication No. 12/2020

ISBN 978-951-51-6056-0 (paperback)
ISBN 978-951-51-6057-7 (PDF)
ISSN 2342-5423 (print)
ISSN 2342-5431 (Online)

Electronic publication available at http://ethesis.helsinki.fi

Printed in Unigrafia OY, Helsinki 2020

CONTENTS

CONTENTS 3

Abstract 5

Acknowledgements 7

List of original publications 8

Authors' contribution 9

Abbreviations 10

1 Introduction 11

1.1 Ensiling of forage legumes 11

1.2 Clostridia 12

1.3 Silage additives 14

2 Objectives and hypotheses of the study 16

3 Summary of material and methods 17

3.1 Experimental forage crops and ensiling procedures 17

3.2 Chemical analyses and aerobic stability 19

3.3 Clostridium analyses 19

4 Results and discussion 21

4.1 Ensilability traits of forage crops 21

4.1.1 Dry matter 21

4.1.2 Water-soluble carbohydrates 23

4.1.3 Buffering capacity 24

4.1.4 Nitrate 24

4.1.5 Clostridia 25

4.1.6 Fermentation coefficient 25

5 Silage quality 28

6 Conclusions 34

7 Future research.....35

8 References 36

ABSTRACT

The objectives of this research were to investigate the ensilability of legume bi-crops and the effect of additives on silage fermentation quality. Silages were made in laboratory scale-silos, and their quality was assessed by qPCR quantification of clostridia DNA and fermentation pattern. Mixtures of white lupin (*Lupinus albus*) and spring wheat (*Triticum aestivum*) were ensiled unwilted at early and late maturity stages (publication **I**) and at late maturity stage both unwilted and wilted (publication **II**). A mixture of red clover (*Trifolium pratense*), timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) was wilted 21 and 45 hours before ensiling (publication **III**). The additive treatments were untreated control (CON), formic acid (FA, 4 L t^{-1} fresh matter), mixtures of sodium nitrite and hexamethylenetetramine (NaHe), and sodium nitrite alone (SN). Lactic acid bacteria (LAB, homofermentative) treatment was only used in **I**.

Dry matter (DM) concentration of forage crops ranged from 199 to 314 g kg^{-1} DM. The ensiled bi-crops in **I** were low in nitrate (0.2 g kg^{-1} DM), while nitrate concentrations in **II** and **III** were 3.8 and 4.0 g kg^{-1} DM, respectively. The water-soluble carbohydrate (WSC) concentration of the late maturity stage mixtures in **I** were 43 and 56 g kg^{-1} DM. The WSC concentration of the other investigated herbages varied from 82.6 (**III**) to 115 g kg^{-1} DM (**II).** The fermentation coefficients (FC) were calculated using DM and WSC concentrations and buffering capacity of pre-ensiled crops to predict the success of preservation without additive treatment. In most cases, FC predicted risk for clostridial fermentation with the FC value ranging between 28.3 (**III)** and 53 (**I)**.

Control and FA treatments produced high butyric acid concentrations of silages in **I**, and lower or zero concentrations in **II** and **III**, whereas NaHe and SN exposed no or only traces of butyric acid. Lactic acid bacteria treatment was successful only with lupin-wheat mixtures having high WSC concentrations at early maturity stage (**I)**. Control treatment exposed high ammonia-N values between 129 and 241 g kg^{-1} N in all investigated lupin-wheat mixtures (**I** and **II**). The number of clostridial DNA copies (spores, vegetative cells and dead cells/spores) was highest in the CON and FA treatments. All silages were aerobically stable (**I-III**).

The effect of hexamine (hexamethylenetetramine) on silage quality was investigated at two DM concentrations of a lupine-wheat mixture (**II**). Hexamine addition did not improve silage quality. Increasing hexamine concentration in a sodium nitrite solution showed no effect on clostridial activity compared to sodium nitrite alone. Clostridia was detected only in a few FA replicate silos (**II**).

A mixture of red clover, timothy and meadow fescue was heavily contaminated with clostridia DNA in both unwilted (log copies g^{-1} 13.3) and

wilted (log copies g^{-1} 9.9) herbage (**III**). Control and SN treatments did not produce butyric acid in either unwilted or wilted silages, while silage butyric acid (2.7 g kg^{-1} DM) was observed in unwilted FA. The clostridial DNA copy numbers were generally high in all silages, and only minor differences between treatments were found.

The silages made of herbage with 3.8-4.0 g nitrate kg^{-1} DM contained no butyric acid or low concentrations of butyric acid below 3 g kg^{-1} DM. The use of SN as a sole solution (900 g^{-1} t) or as a mixture with hexamine (NaHe) produced silages of better quality than the treatments with FA (4 L t^{-1}).

In conclusion, legume bi-crops are difficult to ensile due to low DM, high buffering capacity, low nitrate concentration and being prone to clostridial activity and butyric acid fermentation. Nitrite-based additives were more suitable than formic acid when ensiling legume bi-crops that are prone to clostridial contamination.

Keywords: additive, bi-crop, clostridia, formic acid, meadow fescue, nitrate, qPCR, red clover, silage, sodium nitrite, spring wheat, timothy, white lupin

ACKNOWLEDGEMENTS

I want to express my sincere gratitude to all those who made this work possible:

My supervisor Docent Seija Jaakkola for her continual support and supervision during the years.

Professor Aila Vanhatalo for supporting my work.

Professor Marketta Rinne for supporting my work.

Docent Kari Elo and PhD student Emilia König for the qPCR development and adapting the method for the Department of Agricultural Sciences.

Docent Tuomo Kokkonen for his help on statistical solutions.

Professor emeritus Matti Näsi for his help and support.

Co-authors: Marjukka Lamminen, Kirsten Weiss, Tero Tuomivirta, Sonia Sanz Muñoz, Hannu Fritze, Laura Puhakka for their support and contribution.

Laboratory staff: Leena Luukkainen, Anne Vepsäläinen, Karoliina Heinonen Anne Hannikainen and Ilkka Simpura for their support and contribution.

Funding of the work: Niemi Foundation, Finnish Ministry of Agriculture and Forestry, Future Fund of the University of Helsinki and the Future Fund of the University of Helsinki.

Thanks to my Family.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on work reported in the following original publications, subsequently referred to in the text by their Roman numerals:

I König, W., Lamminen, M., Weiss, K., Tuomivirta, T. T., Munoz, S. S., Fritze, H., Elo, K., Puhakka, L., Vanhatalo, A. & Jaakkola, S. 2017. The effect of additives on the quality of white lupin-wheat silage assessed by fermentation pattern and qPCR quantification of clostridia. Grass and Forage Science 72: 757-771. DOI: 10.1111/gfs.12276

II König, W., König, E., Weiss, K., Tuomivirta, T. T., Fritze, H., Elo, K., Vanhatalo, A. & Jaakkola, S. 2019. Impact of hexamine addition to a nitrite-based additive on fermentation quality, clostridia and Saccharomyces cerevisiae in a white lupin-wheat silage. Journal of the Science of Food and Agriculture 99: 1492-1500. DOI: 10.1002/jsfa. 9322.

III König, W., König, E., Elo, K., Vanhatalo, A. & Jaakkola, S. 2019. Effects of sodium nitrite treatment on the fermentation quality of red clover-grass silage harvested at two dry matter concentrations and inoculated with clostridia. Agricultural and Food Science 28: 155-164.

AUTHORS' CONTRIBUTION

Table 1 describes the contributions of all authors to the original publications of this thesis (author initials are listed in alphabetical order).

Table 1. The contributions of authors to the publications

	Publications		
Phase of work	**I**	**II**	**III**
Planning the experiment	HF, SJ, TT, WK	SJ, WK	SJ, WK
Conducting the experiment	LP, KE, ML, SJ, WK	EK, KE, SJ, WK	EK, KE, SJ, WK
Laboratory analysis	KW, SM, TT, WK	EK, KE, KW, TT, WK	EK, KE, WK
Data analysis	TT, SJ, WK	TT, SJ, WK	EK, KE, SJ, WK,
Drafting the first version of manuscript	WK	WK	WK
Commenting and modifying the manuscript	AV, HF, KE, KW, LP, ML, SJ, TT, WK	AV, EK, HF, KE, KW, SJ, TT, WK	AV, EK, KE, SJ, WK

AV = Aila Vanhatalo
EK = Emilia König
HF = Hannu Fritze
KE = Kari Elo
KW = Kirsten Weiss
LP = Laura Puhakka
ML = Marjukka Lamminen
SJ = Seija Jaakkola
SM = Sonia Sanz Muñoz
TT = Tero Tuomivirta
WK = Walter König

ABBREVIATIONS

BC	Buffering capacity
bp	Base pairs
cfu	Colony-forming unit
CON	Control
DLG	German Agricultural Society (Deutsche Landwirtschafts-Gesellschaft)
DM	Dry matter
DM_{min}	Minimum DM according to Weissbach (1999)
DOMD	Digestible organic matter in dry matter
FA	Formic acid
FC	Fermentability coefficient
FM	Fresh matter
HDM	High dry matter
iNDF	Indigestible neutral detergent fibre
LA	Lactic acid
LAB	Lactic acid bacteria
LDM	Low dry matter
log	Decadal logarithm
mEq	Milli-equivalents
N	Nitrogen
NaHe	Mixture consisting of sodium nitrite and hexamine
NDF	Neutral detergent fibre
NO2	Nitrite
NO3	Nitrate
OM	Organic matter
Osm kg^{-1}DM	Osmol per kg DM
pH	Negative decadal logarithm of the concentration of hydrogen ions
qPCR	Quantitative polymerisation chain reaction
SN	Sodium nitrite
ssp	Species
VFA	Volatile fatty acid
WSC	Water-soluble carbohydrates

1 INTRODUCTION

Ensiling is utilized as a method to preserve many different types of crops. The technology is simple and includes compression of the harvested material followed by airtight sealing. The epiphytic lactic acid bacteria convert free sugars into lactic acid, which increases the silage acidity (decrease pH) to preserving levels.

Different silage additives are used to control the fermentation process to obtain high-quality silage. Virtanen (1933) treated fresh-cut herbage with mineral acids (e.g., hydrochloric acid, sulfuric acid) and realized that mal-fermentation, protein break-down, and cell respiration were the main reasons for low-quality silage. Virtanen (1933) summarized the effects of acidification (1933): "All detrimental breakdown processes in the fodder would be eliminated by treating the fodder, at the time of ensiling, with such amounts of acid as would rapidly raise the acidity of the mass to a point below pH 4.0." Direct acidification of the herbage is called the "AIV- process". Later, the utilization of organic acids, like formic acid (FA), replaced the application of mineral acids due to their high corrosivity, danger to human health, and the need to handle high levels of mineral acid per ton of herbage.

The technological evolution of making silage, e.g., wilting the forage prior to ensiling, made it possible to use lactic acid bacteria (LAB) effectively as silage additive. The use of preserving salts (e.g., sodium nitrite (SN)) was investigated during the 1960s. The utilization of SN has many advantages, such as noncorrosivity and a better effect on suppressing clostridia, when compared with acids. SN's mode of action does not depend on low pH values.

Nitrogen (N)-fixing legumes have an important role in crop rotation, reducing dependence on synthetic N-fertilizer and increasing protein concentration of the ensiled crop. Therefore, there has been a growing interest in preserving legumes. However, legumes are regarded as difficult to ensile and prone to clostridial spoilage because of their low dry matter (DM) content and high buffering capacity (BC) (McDonald et al., 1991).

1.1 ENSILING OF FORAGE LEGUMES

Successful conservation of a forage crop as silage depends on its various ensilability traits. Weissbach (1968) found the connection between DM and water-soluble carbohydrates (WSC) concentrations and BC of the forage plant to be ensiled. An equation was introduced for a so-called fermentation coefficient (FC), which predicts the ensilability of the forage: FC = DM (g kg^{-1})/10 + 8 x WSC (g kg^{-1} DM)/BC (expressed as lactic acid (LA) g kg^{-1} DM) (Schmidt et al., 1971). A fermentation coefficient higher than 45 should predict a butyric-acid free silage

without utilizing a silage additive, which means the WSC/BC ratio of the crop should be 3 or higher to provide enough WSC for lactic acid fermentation and fast acidification. Rearranging the formula to minimum DM (DM_{min}) (g kg^{-1}) = (450 – 80* WSC/BC) gives the result for the minimum DM value of the ensiled crop (Weissbach, 1999). Wilting the crop to the DM_{min} value should predict a butyric acid-free silage.

Anyway, Driehuis and Van Wikselaar's (1996) investigation found butyric acid concentrations of 6 g kg^{-1} DM in grass silages with a DM higher than 600 g kg^{-1}. Weissbach and Haacker (1988) detected butyric acid amounts up to 30 g kg^{-1} DM in whole crop cereal silages wilted to DM concentrations higher than 500 g kg^{-1}. They explained the undesirable butyric acid fermentation as due to a lack of nitrate in the forage crop. According to Kaiser and Weiss (2007), a minimum herbage nitrate concentration of 4.4 g kg^{-1} DM improves FC and predicts butyric acid-free silage.

The osmolality of a solution refers to the concentration of osmotically active particles in that solution. Another approach to explain the occurrence of butyric acid in high DM forages is the quantification and change of forage plants' osmolality during different stages of ensiling (Hoedke, 2007). The DM-dependent osmotic effect (osmol·kg^{-1} DM) reveals the differences between different plant material with the same DM concentration. The fermentation process and maturity stage of the plant have an impact on osmolality (Hoedke, 2007).

The fermentation quality of legume silages is commonly reduced, especially if ensiled unwilted and without additive treatment (Jones et al., 1999; Pahlow et al., 2002; Fraser et al., 2005; Borreani et al., 2009) because of low DM and WSC concentrations and high BC of forage legumes (Pahlow et al., 2002). Bi-cropping legumes with small grain cereals could improve ensilability of the mixture, because whole crop cereals harvested at dough stage typically have DM concentrations between 300 and 400 g kg^{-1} (Jaakkola et al., 2009), and their buffering capacity is low (Bergen et al., 1991).

Utilization of legumes for silage, wilting and contamination of ensiled crops with clostridia poses challenges for silage management. The use of the right silage additive is crucial for the ensiling success and legume silage quality, and thus more detailed information for the efficiency of additives is needed.

1.2 CLOSTRIDIA

Clostridia are gram-positive, sporulating bacteria, that grow under strictly anaerobic conditions and ferment sugars, organic acids or proteins. Their growth is supported by low DM concentration, low WSC concentration and high BC of the crop (McDonald et al., 1991). Clostridia can be divided into two major groups based on their substrates. Saccharolytic clostridia, for example, *Clostridium butyricum,* mainly ferment carbohydrates. Proteolytic clostridia like *C. sporogenes* ferment

amino acids. The most abundant clostridia species in silage is *C. tyrobutyricum*, which utilizes carbohydrates but can ferment lactate and is very acid tolerant (Driehuis & Oude Elferink, 2000; Driehus, 2013).

Soil, old plant parts and decaying plants in contact with soil, and manure are the sources for clostridial contamination of silage (Ercolani, 1997; Pahlow et al. 2003). Silage that has been subject to clostridial fermentation is called anaerobically unstable (Pahlow et al., 2003). Clostridial fermentation causes energy and DM losses in silage and, therefore, negatively affects animal feed intake and performance. Clostridial spores germinating in milk are responsible for the so-called late-blow effect when fermentation gases destroy the cheese texture (Vissers et al., 2007).

Clostridial fermentation has a positive effect on other spoilage microbes that benefit from higher silage pH values triggered by butyric acid formation. The fermentation of two moles lactic acid to one mole butyric acid (plus hydrogen and carbon dioxide) raises the pH of the silage because butyric acid is the weaker acid (Pahlow et al., 2003).

$$2 \text{ lactic acid} \longrightarrow 1 \text{ butyric acid} + 2H_2 + CO_2$$

Many silage clostridia and enterobacteria can reduce nitrate to ammonia. Enterobacteria reduce nitrate in the first step to nitrite and in a second step to dinitrogen oxide and ammonia. Nitrite reacts in acidic surroundings chemically to nitric oxide and nitrate. Nitric oxide and dinitrogen oxide are toxic to clostridia (Spoelstra, 1985). The antimicrobial properties of nitrous gases, especially nitric oxide, are well known (Spoelstra, 1985; Lück and Jager, 1995; Kaiser and Weiss, 1997).

Clostridia use nitrate as an electron acceptor. The reduction potentials (NADH) are regenerated by reducing nitrate to ammonia. Substrate level phosphorylation provides clostridia with additional ATP (Keith et al., 1982). Reducing nitrate to ammonia increases silage pH due to proton consumption during the reduction process (Spoelstra, 1985). A raise in the silage pH value may enable the activity of other detrimental microbes and reduce silage quality.

Understanding and controlling the function of clostridia requires accurate methods for the determination of microbes. The qPCR method has not been widely used in ensiling studies so far, but PCR-based methods offer a fast and sensitive methodology for a wide range of applications; e.g. these methods can be utilized to detect spoilage microorganisms in silage and milk (Cremonesi et al., 2012).

1.3 SILAGE ADDITIVES

Formic acid

The effect of FA on silage fermentation is based on direct acidification and antimicrobial properties. The antimicrobial effect of the undissociated FA molecule is the weakest within the aliphatic fatty acid series due to the low pKa-value, which increases with increasing fatty acid chain length (Woolford, 1975). The undissociated FA molecule penetrates the cell wall and dissociates again in the cell, causing a pH decrease (Lambert and Stratford, 1999). When utilizing FA as silage additive, the ensiling success depends on plant pre-ensiling characteristics and the application level of FA. Acidification results in an immediate pH decrease that causes cell wall damage and lysis of the cells. Formic acid is chemically a weak acid, and plants with high BC demand higher application rates than fresh and easy-to-ensile crops (McDonald et al., 1991, p. 198).

Effects of FA on fermentation patterns of crops easy-to-ensile are high residual WSC concentrations and restricted proteolysis. Formic acid also has a restricting effect on lactobacteria growth (McDonald et al., 1991 p. 202). Yeasts are known to be tolerant to FA, and high amounts of ethanol can be found in FA-treated silage (Henderson et al., 1972).

The experiment of Rammer (1996) showed that FA had no anticlostridial effect when grass herbage was infected with spores of *C. tyrobutyricum* and ensiled with FA (85%) 4 ml kg^{-1} FM. Yingxi (2016) found that the effect of FA is weak against *C. tyrobutyricum*. A formic acid (85%) application rate of 4 ml kg^{-1} FM did extend the lag phase of *C. tyrobutyricum*, but there was no difference in the yield of butyric acid compared with the control. The application rate corresponded to the commonly used amount of FA while ensiling forage in Finland. According to Huhtanen et al. (2012) formic acid turned into the most used silage additive in Finland. This leads to the question of whether formic acid is also effective in ensiling different types of forage legumes.

Nitrite-based additives

Hellberg (1967) started to investigate a mixture of sodium nitrite and hexamine as a silage additive. The anticlostridial effect of nitrite has been known for centuries (Lück and Jager, 1995). Wieringa (1958) investigated the inhibition of butyric acid fermentation by nitrite. He found that nitrite as a reduction product of plant nitrate suppressed butyric acid fermentation in grass silages; he concluded that a plant nitrate concentration of 6 to 10 g NO_3 kg^{-1} DM produces better silage quality than assumed from the chemical composition of the herbage.

The antimicrobial effect of nitrite is based on the released nitric acid and the emerging nitrogen oxides. Nitric oxide penetrates the bacteria cell wall and inhibits e.g. glycolysis catalyzing enzymes. The antimicrobial effect of nitrites increases with

decreasing pH (Lück and Jager, 1995). Hexamine and nitrite in mixtures with sodium benzoate and sodium propionate is utilized to improve anaerobic and aerobic stability of silage (Lättemäe and Lingvall, 1996; Lingvall and Lättemäe, 1999). Sodium nitrite, sodium benzoate and potassium sorbate were utilized as additives in varying compositions to evaluate the ensiling effect on a mixture of red clover, timothy and meadow fescue (Knicky and Spörndly, 2009). All mixtures improved silage quality and storage stability.

Although nitrite and hexamine have been used as silage additives because of their adverse effects on clostridia (Hellberg, 1967), little research is done on that subject under the growing and ensiling conditions in Finland. In addition, no research is available on the comparison between sole sodium nitrite and sole formic acid in preventing clostridia in preserving difficult-to-ensile legume forages.

Lactic acid bacteria

Lactic acid bacteria can be roughly divided into two classes according to their fermentation products from glucose. If lactic acid is the main fermentation product LAB is called homolactic and heterolactic when various fermentation end products are formed (Kung et al., 2003). Lactic acid bacteria applied as silage additive should rapidly grow under various environmental conditions, be acid-tolerant, utilize different WSC, dominate epiphytic organisms and be homofermentative (Wieringa and Beck, 1964). A rapid decrease in silage pH inhibits clostridial growth and plant protein degradation (Kung et al., 2003). The ensiling success depends on WSC concentration of the crop and the amount of applicated lactic acid bacteria. Heterolactic LAB like *L. buchneri* are utilized to improve silage aerobic stability of high DM forages (Kleinschmid et al., 2006).

2 OBJECTIVES AND HYPOTHESES OF THE STUDY

The experiments in the thesis aimed to study the efficacy of different additives (formic acid, a mixture of sodium nitrite and hexamine, sole sodium nitrite, and inoculant containing lactic acid bacteria) when ensiling legume-based forages. The specific objective was to compare the efficiency of sodium nitrite and formic acid used at an application rate of 4 L t^{-1} FM against clostridia. The efficacy of additives was assessed by analyzing silage fermentation quality and prevalence of clostridial species. Quantitative polymerization chain reaction (qPCR) was used to assess different clostridia by their DNA-copies.

The ensiling trials were arranged to achieve variable ensilability traits of forage crops by changing plant species ratios, using different wilting times and harvesting the crops at different maturity stages. The ensiled crops were white lupin-wheat mixtures having different maturity stages, proportions of white lupin and DM concentrations, and red clover-grass mixtures having different DM concentrations. Forage crops having different ensilability traits were studied in separate sub-trials and thus tested only in terms of the effects of the additives. Therefore, it was not possible to statistically test whether the additives had a different effect when the ensilability traits varied.

The main hypotheses tested in this thesis are:

1) The use of additives compared to untreated control leads to an overall improvement in silage quality, e.g. by preventing clostridial and yeast fermentation (I, II, III)

2) Chemical additives are more effective than lactic acid bacteria in improving silage quality (I)

3) A mixture of sodium nitrite and hexamine or sole sodium nitrite are more effective than formic acid (4 L t^{-1} FM) in preventing secondary fermentation and proliferation of most commonly occurring clostridial species in silages (I, II, III)

4) Adding increasing amounts of hexamine with sodium nitrite suppresses clostridia proliferation in silage (II)

Roman numerals in brackets refer to the three publications

3 SUMMARY OF MATERIAL AND METHODS

This thesis comprises three publications (I, II and III) with four separate sub-experiments in I, two separate sub-experiments in II as well as in III. All experimental silages were produced at the Viikki Research Farm of the University of Helsinki, Finland (60° N, 25° E). Materials and methods are only briefly described because they are explained in detail in the original publications (I–III). A brief summary of the trials is presented in Table 2.

3.1 EXPERIMENTAL FORAGE CROPS AND ENSILING PROCEDURES

For the research paper I, a mixture of white lupin (*Lupinus albus*, variety Ludic) and spring wheat (*Triticum aestivum* L., var. Amaretto) was harvested at two stages of maturity. White lupin was separated from the wheat and both plants were chopped using a laboratory chopper. After that, two mixtures of white lupin and spring wheat were reformed for ensiling at both maturity stages. The plot area was fertilized with an artificial fertilizer 60 kg N ha^{-1} at sowing in the spring. A bi-crop of white lupin (var. Feodora) and spring wheat (var. Amaretto) was used in II for two separate experiments ensiled either unwilted or after 40 h wilting time. The field was fertilized in the previous autumn with livestock manure and in spring with an artificial fertilizer, resulting in a total of 50 kg N ha^{-1}. In research paper III, the study comprised two ensiling experiments. The field area used was a second-year legume-grass mixture of red clover (*Trifolium pratense*), timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*). The field was not fertilized in the spring. More details for the ensiled crops used in I-III is given in Table 2.

The herbages were cut at a stubble height of about 10 cm either with electric scissors (I) or by utilizing a disc mower (Krone EasyCut 3210 CV, Maschinenfabrik Bernard Krone GmbH, Spelle, Germany) (II, III). Before ensiling the herbages were chopped using a laboratory chopper (Wintersteiger, Ried im Innkreis, Austria) to give a chop length of 1–4 cm.

The forages were ensiled in 1.5 L glass silos (Weck, Wher-Oflingen, Germany) with three (I, II) or four (III) replicates per treatment. Silos were fitted with a lid with a rubber seal, which enabled the release of fermentation gases. All silos were stored at ambient room temperature (20–22°C) and opened 100 and 101 days (I), 154 days (II) and 106 days (III) after ensiling. In II, the same additive-treated herbages as ensiled in 1.5 l silos were also ensiled in glass silos with a volume of 120 mL to study the effect of additives on silage pH in the early phase of ensiling. These silos were sealed with a rubber stopper and a screw cap. For each treatment, eight replicate silos were used.

Table 2. The summary of publications and experiments.

Publ.	Exp.	Plant species	Proportion of white lupin/red clover g kg^{-1} on FM basis	Growth stage	Wilting
I	1	White lupin (*Lupinus albus*, 'Ludic') + spring wheat (*Triticum aestivum L.*, 'Amaretto')	333	Cut 14th of August 2012, 96 DAS: lupin pods filled to 50% with green seeds; wheat at the beginning of dough stage	Unwilted
	2		666		Unwilted
	3		333	Cut 28th of August 2012, 110 DAS: lupin pods filled to 75% with green seeds; wheat at the end of dough stage	Unwilted
	4		666		Unwilted
II	1	White lupin (*Lupinus albus,* 'Feodora') + spring wheat (*Triticum aestivum L.*, 'Amaretto')	700	Cut August 16th and 19th 2014, 89 and 92 DAS: lupin pods filled to 75% with green seeds; wheat at the end of dough stage	Unwilted
	2		700		Wilted 40 h
III	1	Red clover (*Trifolium pratense*) + timothy (*Phleum pratense*) + meadow fescue (*Festuca pratensis*)	659	Cut 9th of August 2016 as a second cut of the summer, at the beginning of flowering of red clover	Wilted 21 h
	2		659		Wilted 45 h

FM, fresh matter; DAS, days after sowing

A summary of the additives used in the experiments given in Table 3. In each trial, to ensure even distribution silage additives were applied as a water solution with a volume of 10 mL kg^{-1} fresh matter (FM), including additive and water. For the control (CON) silages (I, II, III), the herbage was treated with 10 mL kg^{-1} FM tap water. In I, the additives used were FA (Sigma-Aldrich, St. Louis, MO, USA), a mixture of SN and hexamine (NaHe) (Kofasil Liquid; Addcon, Bonn, Germany) and homofermentative LAB (dosage 100 000 cfu g^{-1} forage) (Agrosil Premium, manufactured by Addcon, Bonn, Germany). In II, the forages were treated with FA and three mixtures of SN (Sigma Aldrich, St Louis, USA) and hexamine (Sigma Aldrich, St Louis, USA) (NaHe). In III, half of the silos were filled with herbage batch inoculated before additive treatment with *C. tyrobutyricum* produced for the trial (Bionautit, Helsinki, Finland). The inoculation solution was spread with a pipette while herbage was simultaneously thoroughly mixed. Immediately after that the herbage was treated in the same way with FA or with sodium nitrite.

3.2 CHEMICAL ANALYSES AND AEROBIC STABILITY

In all trials, representative samples were collected from the experimental field areas before harvesting for botanical analyses and from the chopped herbage before ensiling for DM, ash, crude protein, soluble N, neutral detergent fibre (NDF), WSC, nitrate, buffering capacity, *in vitro* pepsin-cellulase solubility, clostridia (I-III) and yeasts only in III. After opening the silos, samples were taken for analysis of pH, fermentation characteristics, aerobic stability, clostridia (I-III) and yeasts (III).

After opening the silos, aerobic stability of the silages was measured by recording the temperature every 5 minutes over 12 days (data loggers MicroLite, Fourier Systems Ltd, USA). Aerobic stability was expressed as time elapsed until the temperature rose to 2°C over the mean ambient temperature (20–22°C).

3.3 CLOSTRIDIUM ANALYSES

The qPCR analyses of four Clostridium species (*C. butyricum*, *C. tyrobutyricum*, *C. sporogenes*, and *C. perfringens*) were conducted in the laboratory of the Natural Resources Institute of Finland (Luke) (I, II) and in the laboratory of University of Helsinki (III).

The length of the PCR products varied from 254 to 285 base pairs (bp). The absolute quantification was achieved by interpolation of standard curves. The used DNA extraction protocol should extract DNA from bacterial endospores and cells. The gene copy number is known to vary from 1 to 15 per bacterial genome depending on bacterial species (Stoddard et al., 2015). The detection limit for qPCR was approximately 2,000 gene copies per gram of herbage or silage, which equals to approximately 200 bacterial cells or endospores per gram FW.

Table 3. The summary of additives used in the experiments.

Publ.	Exp.	Additive treatment	Abbreviation	Additive/DSM number	Application rate of effective substance (as 100%)
I	1-4	Control	CON	No additive	-
		Formic acid	FA	CH_2O_2 (950 g kg^{-1})	Formic acid 4 L t^{-1} FM
		Sodium nitrite + hexamine	NaHe	$NaNO_2$ and $C_6H_{12}N_4$	Sodium nitrite 750 g t^{-1} FM+hexamine 500 g t^{-1} FM
		Lactic acid bacteria	LAB	*L. plantarum* 3676 and 3677	1 x 10^6 cfu g^{-1} FM
II	1-2	Control	CON	No additive	-
		Formic acid	FA	CH_2O_2 (950 g kg^{-1})	Formic acid 4 L t^{-1} FM
		Sodium nitrite	NaHe0	$NaNO_2$	Na-nitrite 900 g t^{-1} FM
		Sodium nitrite + hexamine	NaHe300	$NaNO_2$ + hexamine	Na-nitrite 900 g t^{-1} FM + hexamine 300 g t^{-1} FM
		Sodium nitrite + hexamine	NaHe600	$NaNO_2$ + hexamine	Na-nitrite 900 g t^{-1} FM + hexamine 600 g t^{-1} FM
III	1-2	Control	CON	No additive	-
		Formic acid	FA	CH_2O_2 (950 g kg^{-1})	Formic acid 4 L t^{-1} FM
		Sodium nitrite	SN	$NaNO_2$	Na-nitrite 900 g t^{-1} FM
		CON + clostridia		*C. tyrobutyricum*	1 x 10^5 cfu g^{-1} FM
		FA + clostridia		*C. tyrobutyricum*	1 x 10^5 cfu g^{-1} FM
		SN + clostridia		*C. tyrobutyricum*	1 x 10^5 cfu g^{-1} FM

cfu, colony-forming unit

4 RESULTS AND DISCUSSION

4.1 ENSILABILITY TRAITS OF FORAGE CROPS

The characteristics of the herbages before ensiling are shown in Table 4. A wide variation was attained in DM concentration and other ensilability traits of the investigated forage crops when composed of two re-formed mixtures of white lupin and spring wheat at two growth stages (I), a sown unwilted and wilted mixture of white lupin and spring wheat (II) and a sown, wilted mixtures of red clover and grass at two DM levels (III).

The ensilability of plant material depends on its chemical, physical and biological characteristics as described e.g., by Jänicke (2011). Varying epiphytic bacteria colonization on the plant is an example for biological characteristics, while DM, chop length and osmotic pressure stand for physical qualities. The chemical composition of the ensiled crop is described through BC, and WSC, crude protein and nitrate concentrations (Jänicke, 2011). In general, the fermentability of forage crops depends on DM, WSC and nitrate concentrations and buffering capacity. Even though these traits are plant-specific, they can be influenced by plant species, plant variety and soil N fertilization (Spolders, 2006). In addition to beneficial ensilability characteristics of crops wilting, clean-cut and suitable silage additives also improve the fermentation process.

4.1.1 DRY MATTER

Due to the differences in plant species, growth stages and wilting, the DM concentration of the pre-ensiled forages ranged from 150 to 314 g kg^{-1}. The high proportion of wheat (666 g kg^{-1} FM) raised the DM concentration of white lupin-wheat mixtures in average 70 g kg^{-1} (I) whereas 40 h wilting time increased DM concentration of the white lupin-wheat mixture 90 g kg^{-1} (II). Increasing wilting time by 24 h (from 21 to 45 hours) increased red clover-based herbages DM concentration by approximately 115 g kg^{-1} (III).

Forage DM concentration is correlated to osmolality because the removal of water increases osmo-active particles per kg forage. This, in turn, increases the relative WSC concentration and improves crop ensilability compared with the fresh crop. Osmolality refers to the number of solute particles in 1 kg of solvent. Because water is the solvent, and the osmo-active particles are diluted in water, osmolality is expressed as millimoles per kg water (Koeppen and Stanton, 2019).

Table 4. Chemical composition and ensilability traits of whole crop white lupin (L) and wheat (W) mixtures (publications I and II) and red clover-based herbage (publication III) (g kg^{-1} dry matter (DM) unless otherwise stated).

	Publication I				Publication II		Publication III	
	MIX 1	MIX 2	MIX 3	MIX 4			Wilted	Wilted
	Growth stage 1		Growth stage 2		Unwilted	Wilted	low DM	high DM
Proportion of lupin/red clover, g kg^{-1}	333	666	333	666	700	700	659	659
Dry matter, g kg^{-1}	307	235	285	212	150	240	199	314
Calculated DM_{min}, g kg^{-1}	231	262	345	343	304	294	366	345
Ash	91.6	84.2	85	79.7	73.9	70.4	88.3	83.3
Crude protein	91	123	81	114	171	151	188	177
Soluble N, g kg^{-1} N	605	520	570	502	487	699	367	318
Neutral detergent fibre	497	460	510	486	437	499	460	467
Water soluble carbohydrates (WSC)	91	103	43	56	115	111	82.6	95.7
WSC, g kg^{-1} FM	28	24	12	12	17.2	26.6	16.4	30.1
Starch	152	113	218	154	52.7	87.6	-	-
In vitro digestible organic matter	587	599	586	591	650	643	-	-
Buffering capacity (BC)								
mEq kg^{-1} DM	370	488	359	466	703	630	872	805
Lactic acid, g kg^{-1} DM	33.3	44	32.3	42	63	57	78.6	72.6
Nitrate	<0.2	<0.2	<0.2	<0.2	3.8	3.8	4	4
Fermentation coefficient (FC)	53	42	39	32	29.6	39.6	28.3	42
Microbes, log copies g^{-1} FM								
Sum of clostridia	ND	ND	ND	ND	5.3	9.61	13.3	9.9
Saccharomyces cerevisiae	NA	NA	NA	NA	7.43	6.81	NA	NA

Calculated DM_{min}=450 + 80 x WSC (g kg^{-1} DM)/BC (g kg^{-1} DM) (Weissbach, 1999); FC=DM (g kg^{-1})/10 + 8 x WSC (g kg^{-1} DM)/BC (g kg^{-1} DM) (Schmidt et al., 1971)

ND, not detected; NA, not analyzed

Herbage osmolality has a direct impact on microbe's osmotolerance and silage quality. Increased osmolality impairs growth of microbes (Rojas and Huang, 2018). Thus, the actual reason for the inhibition of clostridia in wilted herbage is the increasing osmolality.

According to Hoedke (2007), e.g. WSC, amino acids, alcohols and mineral ions increased the osmolality of the plants, whereas starch, and other macromolecules decreased the osmolality due to their high mole masses. This induces that the crop osmolality varies during the growing process if WSC is used to build starch and amino acids are used for proteins. This suggests that, e.g. decreasing WSC and increasing starch concentration between growth stages decreased osmolality of white lupin-wheat mixtures (I).

4.1.2 WATER-SOLUBLE CARBOHYDRATES

Water-soluble carbohydrate concentration of pre-ensiled crops varied in the experiments from 12 to 30 g kg^{-1} FM. In unwilted bi-crop mixtures at later maturity stage (I) WSC concentration was low (12 g kg^{-1} FM), because both lupine and wheat are low in WSC due to starch formation (DLG, 2011). In II, the WSC concentration in DM basis was at the same level in both forages, whereas when expressed in FM basis wilting increased the WSC concentration of bi-crop from 17.2 to 26.2 g kg^{-1}. Similarly, on FM basis, the WSC concentration was almost twice as high in high DM as in low DM red clover-based herbage (III).

Water-soluble carbohydrates (glucose, fructose, sucrose, fructans) are substrates for silage fermentation. A minimum WSC concentration of 25-30 g kg^{-1} FM has been suggested to be necessary for a sufficient acidification of the forage crop without additive treatment (Wilkins, 1983; Pettersson, 1988). Accordingly, EFSA (2006) categorized forages easy to ensile if WSC concentration is higher than 30 g kg^{-1} FM (e.g. whole plant maize, ryegrass) and difficult to ensile if WSC is lower than 15 g kg^{-1} FM (e.g. leguminous plants). Thus, in the present experiments, the forage crops were mainly difficult or moderate difficult to ensile based on WSC concentration. The only exception was wilted red clover-based herbage with 30.1 g WSC kg^{-1} FM (III).

Fructans are soluble storage carbohydrates of temperate grasses while in legumes storage (structural) carbohydrate is starch. Starch is higher polymerized than fructans and, therefore, without enzymatic actions or hydrolysis, is not directly available as a substrate for micro-organisms. Legumes accumulate carbohydrates as starches; thus, their WSC concentration is smaller than those of temperate grasses (McDonald et al., 1991). In the present experiments, starch concentration of white lupin and wheat (I) varied between the two maturity stages. The starch concentration of wheat increased from 183 to 255 g kg^{-1} DM, while the starch concentration of white lupin was almost the same at maturity stages 1 and 2 (30 and 23 g kg^{-1} DM).

4.1.3 BUFFERING CAPACITY

Buffering capacity is the resistance of the plant species to natural acidification (McDonald and Henderson, 1962) and defined as the amount of lactic acid required to adjust pH of the fresh material to 4.0 (Weissbach, 1992). The buffering capacity is influenced by plant species, N-fertilization, maturity state and clostridial contamination. The neutralizing effect is mainly attributed to the concentration of salts of organic acids (Playne and McDonald 1966).

Legumes contain more crude protein and are richer in organic acids, resulting in higher buffering capacities (McDonald and Henderson, 1962). The corresponding mean buffering capacities were for white lupin 62.9 g LA kg^{-1} DM and for wheat 26.8 g LA kg^{-1} DM in I. Accordingly, the buffering capacity of the white lupin-wheat mixture increased from 32.8 to 43.0 g LA kg^{-1} with an increasing proportion of white lupin. However, only minor differences were observed between growth stages (I). The buffering capacity of the bi-crop was higher (mean 60 g LA kg^{-1}) in II than in I regardless of the low buffering capacity of wheat. The higher crude protein and lower starch concentration of bi-crop in II than in I suggest a higher proportion of white lupin in the bi-crop than was measured in the botanical analyses. This may explain the difference in buffering capacity between the experiments.

Playne and McDonald (1966) found that wilting reduces the concentration of organic acids; therefore, buffering capacity decreases. This is in concordance with the findings of this work in II and III (Table 4). According to Jänicke (2011), the buffering capacity of red clover varies between 69 and 80 g LA kg^{-1} DM and that of grass between 38 and 60 g LA kg^{-1} DM.

4.1.4 NITRATE

The ensiled bi-crops' nitrate concentration was below 0.2 g kg^{-1} DM (I) in the present experiments, the same for both the wilted and unwilted bi-crops (3.8 g kg^{-1} DM) (II) and the same for both LDM and HDM red clover-based herbage (4.0 g kg^{-1} DM) (III). Thus, all the crops used in this work had nitrate values below 4.4 g kg^{-1} DM which has been suggested to be a minimum amount for butyric-free silage (Kaiser and Weiss, 2007).

Plant nitrate concentration increases with increasing N fertilization. Furthermore, the application time and the amount of nitrogen affects plant WSC concentration (Podkowka, 1969; Fiebig et al., 1974). A moderate N fertilization favors the synthesis of carbohydrates and nonprotein N compounds (Wilman, 1980), while increased N amounts raise the amounts of amino acids and amines and decrease WSC concentration (Mengel, 1991). The difference in nitrate concentration of white lupin-wheat mixture between I and II was probably caused by a different type of N-fertilization.

Forage grasses, cereal grain crops and legumes are weak nitrate-storing plants. Pursiainen and Tuori (2008) found nitrate values of 0, 0.24, 0.24-0.60 and 1.2-2.4 g kg^{-1} DM for whole crop field bean, field pea, common vetch and wheat, which correspond with the results of white lupin and wheat in I. Atkins

et al. (1975) found that asparagine is the major assimilation product of nitrogen fixation and nitrate reduction in many legumes and the main nitrogenous compound exported from root to shoot. This might explain why legumes are low on nitrate.

Nitrate-rich feeds contain more than 10 g nitrate kg^{-1} DM, while concentrations up to 5 g kg^{-1} DM are regarded as harmless. Ensiling can significantly reduce forage nitrate levels (20-30%) (Spolders, 2006). The nitrate concentrations of the ensiled crops in each experiment were below 5 g kg^{-1} DM and are, therefore, harmless for animals.

4.1.5 CLOSTRIDIA

Quantitative PCR analyses did not detect any of four studied clostridial species in either of the mixtures or white lupin and wheat samples in the first experiments (I). Thus, it is probable that all pre-ensiling samples contained clostridial bacteria and/or spores in amounts below the detection limit of the utilized qPCR method, i.e., less than 200 vegetative bacteria or endospores per gram of sample of each studied Clostridium species. This is concordant with the estimate of Pahlow et al. (2003) that plants typically contain 100–1000 clostridial endospores (cfu g^{-1} FM of crop) prior to ensiling. However, a higher contamination that was observed in the later study with a white lupin-wheat mixture (II) maybe due to manure spreading during the previous autumn. The herbage used in I was fertilized with artificial fertilizers, and no contamination with clostridia was detected.

In red clover-based wilted herbage (III) the LDM forage contained 13.3 log copies g^{-1} FM and the HDM forage 9.9 log copies g^{-1} FM of clostridia ssp. The reason for the herbage contamination with clostridia might be the problems with vast flocks of Canada geese (*Branta canadensis*) spoiling the research area with their droppings.

4.1.6 FERMENTATION COEFFICIENT

The estimation of herbage fermentability is important for the ensiling success in terms of effects on fermentation processes, wilting and the requirement of silage additives. Fermentation coefficient based on buffering capacity and concentrations of DM and WSC of forage crops was used to predict the ensiling success in the present experiments. Increasing DM and WSC concentration raise FC, whereas increasing buffering capacity hampers ensiling.

Legumes are considered to be difficult to ensile because of their low DM concentration, high buffering capacity, and low nitrate concentration (Spolders, 2006). Mixing forage legumes with whole crop cereals generally improves ensilabilty compared with the pure legumes (Pursiainen and Tuori 2008). The proportions of white lupin in I were either 333 or 666 g kg^{-1} FM at both growth stages. The ensilability traits of ensiled crops were impaired by increasing the proportion of white lupin in the bi-crop due to the lower DM

concentration and higher buffering capacity in white lupin than in wheat (Table 4). A higher FC of the white lupin-wheat mixture with a higher proportion of wheat reflected this connection (I). The proportion of white lupin in the bi-crop (700 g kg^{-1} FM) in II was close to the proportion in Mixture 2 in I. The calculated FC was 29.6 in the unwilted and 39.6 in the wilted bi-crop (II).

Red clover affects silage fermentation quality through its ensilability characteristics as shown, e.g., by Dewhurst et al. (2003). Red clover was the dominating part in the herbage used in III, the proportion being 659 g kg^{-1} FM before harvesting. Both red clover-grass mixtures exposed relatively low WSC concentrations, high buffering capacities and FC below 45. The herbage was contaminated with clostridia, as in II. The WSC concentration was slightly higher and BC lower in HDM than LDM herbage. Wilting improved the FC of the forage in the present experiment from 28 (LDM) to 42 (HDM).

Fermentation coefficients greater than 45 should predict butyric acid-free silages, provided that the herbage contains nitrate at least 4.4 g kg^{-1} DM (Kaiser and Weiss, 1997). Considering that none of the herbages used in the present experiments met the nitrate requirement, the DM_{min} and FC values had to be corrected according to Kaiser and Weiss (2007) by increasing the DM requirements for the prediction of butyric acid-free silage (Table 5). The recalculated corrected DM_{min} values did not predict any butyric acid-free silage in I, II, and III (Table 5).

Summarizing all the parameters for good quality silage, the characteristics of white lupin and white lupin-wheat mixtures and red clover-grass mixtures were not destined to obtain good preserving results without any silage additive.

Table 5. Corrected minimun dry matter (DM_{min}) concentration and fermentation coefficient (FC) of experimental forage crops, adapted from Kaiser and Weiss (2007).

Publication	Crops	Schmidt et al. (1971) Weissbach (1999)			Kaiser and Weiss (2007)	Adapted from Kaiser and Weiss (2007)
		DM	DMmin	FC	Corrected DM_{min}	Corrected FC
I	Mix 1	307	231	53	473	69
	Mix 2	235	262	42	501	69
	Mix 3	285	345	39	573	68
	Mix 4	212	343	32	573	68
II	Unwilted	150	304	30	333	48
	Wilted	240	294	40	317	47
III	Low DM	199	366	28	411	50
	High DM	314	345	42	399	50

Mix 1-4, mixtures of white lupin and wheat

DM, dry matter (g kg^{-1})

DM_{min} (g kg^{-1}), minimum DM requirement for butyric acid-free silage (Weissbach, 1999)

FC, fermentation coefficient (Schmidt et al., 1971)

Corrected DM_{min} and FC, adapted from Kaiser and Weiss (2007):

Nitrate < 4.4g NO_3/kg DM: 1) DM_{min} (%) = 68 - 6.4 x NO_3 - 7.1 x WSC/BC if low clostridia contamination

2) DM_{min} (%) = 100 – 11.3 x NO_3 - 13 x WSC/BC if high clostridia contamination

These DM_{min} results replace the DM in FC = DM% + 8 WSC/BC

5 SILAGE QUALITY

Control silage

Table 6 presents the fermentation pattern of silages (I, II, III). Aerobic stability was not affected by additives. All silages were stable during the entire measuring period in every experiment.

Calculated ensilability values FC and DM_{min} predicted butyric acid-free control silage only when white lupin-wheat was harvested at the early growth stage and ensiled with a low proportion of white lupin (I). However, the prediction was wrong since the butyric acid concentration of this silage was very high (38 g kg^{-1} DM). On the other hand, the pre-ensiling requirements for high-quality silage were not fulfilled in any experimental crop (I-III) when DM_{min} was corrected according to Kaiser and Weiss (2007). All control silages contained butyric acid in I, while the prediction was not realized in II and III as expected: only traces or no butyric acid were observed in untreated silages. Consistent with the results in I, untreated field bean silage was badly preserved as evidenced by a high pH, high butyric acid and ammonia-N concentrations and high clostridial spore counts in the experiment of Pursiainen and Tuori (2008).

Ammonia-N concentrations over 120 g kg^{-1} N were measured in all control bi-crop silages in I and II. However, elevated ammonia-N concentration was not always associated with high butyric acid concentration (Figure 1). Butyric acid was found in all sub-experiments of I, although the pH of control silages in sub-experiments 3 and 4 had pH values of 4 and below. Some studies have shown that a pH of 4.2 or even higher would inhibit clostridia activity (Jonsson, 1989). Silage pH of 3.93 did not prevent clostridia activity in I, but in II and III clostridia was inhibited at pH values from 3.92 to 4.12.

The reasons for the different pH values which limited clostridia growth in I compared to II and III might be the different nitrate concentrations in the ensiled crops (Weiss, 2001). During the first ensiling phases, enterobacteria reduce nitrate to nitrite, which is toxic to clostridia (Spoelstra, 1985). Clostridia also utilize nitrate as an electron sink during the regeneration of reduction potentials (nicotine amide adenine dinucleotide) (NADH to NAD^+). Acetic acid is formed instead of butyric acid (Spoelstra, 1985). The low nitrate concentration of the crop and the absence of *C. sporogenes* (amino acids utilizing clostridia species) in 3 experiments (I) suggest that there is little contribution of ammonia-N originated from bacterial reduction of nitrate to ammonia-N. The explanation for the elevated ammonia values of the control silages could be the degradation of proteins by plant enzymes and, finally, by enterobacteria (McDonald et al., 1991, p. 117). The most abundant clostridia species was *C. tyrobutyricum,* which is known to be tolerant of low pH conditions (Pahlow et al., 2003).

Table 6. Summary of fermentation parameters of experimental silages (I – III).

		DM	pH	Amm-N	Amm-N cor	WSC	LA	AA	BA	Eth	Ethyl esters
Publ. I											
Exp.1	CON	290	4.53	188	188	29.2	32.5	3.24	37.7	25.2	170
	FA	311	4.28	66	66	109	2.9	4.59	6.01	3.78	24
	NaHe	313	5.01	107	25	113	17.2	10.48	0	2.32	31
	LAB	309	3.75	62	62	23.9	53.0	4.75	0	6.37	171
Exp.2	CON	226	4.60	241	241	14.2	45.9	7.23	43.1	28.3	315
	FA	237	4.06	54	54	87.2	24.9	6.79	9.32	5.92	0
	NaHe	240	4.67	127	37	112	38.4	11.71	0.54	5.42	27
	LAB	245	3.83	60	60	21.3	75.3	7.27	0	12.9	439
Exp. 3	CON	315	4.05	129	129	12.7	41.9	6.37	4.44	5.8	207
	FA	311	4.69	128	128	33.6	3.8	4.85	11.6	3.99	0
	NaHe	317	4.20	124	36	39.1	32.7	9.36	0	1.61	0
	LAB	327	4.08	107	107	15.0	34.7	4.26	5.69	4.35	69
Exp. 4	CON	218	3.93	155	155	15.3	70.4	10.3	5.16	11.3	371
	FA	226	4.20	112	112	65.9	5.1	8.3	8.7	3.41	0
	NaHe	229	3.96	138	44	30.5	57.8	11.7	0.43	3.57	0
	LAB	245	4.00	130	130	13.4	45.8	13.4	6.51	7.55	178
Publ. II											
Exp. 1	CON	140	3.83	138	138	15.7	120	23.9	0.23	14.3	391
	FA	143	3.75	50	50	208	0	8.8	0.00	1.53	0
	NaHe0	154	3.86	141	89	11.2	119	19.3	0	6.68	259
	NaHe300	156	3.95	175	83	13.7	111	22.1	0	9.65	324
	NaHe600	138	4.08	204	89	18.9	102	25.3	0	18.2	530
Exp.2	CON	219	3.92	157	157	21.5	91.8	18.5	0.26	7.18	294
	FA	236	3.90	99	99	33.7	44.8	13.0	1.57	9.79	267
	NaHe0	235	3.94	136	101	20.1	86.2	17.3	0.33	2.68	159
	NaHe300	231	4.03	156	96	31.4	82.1	16.8	0	2.12	106
	NaHe600	217	4.18	176	98	57.6	72.4	14.4	0.43	3.13	150
Publ. III											
Exp. 1	CON	213	4.08	81	81	4.3	133	36.1	0	3.3	
	FA	204	4.08	46	46	90.2	23	9	2.7	5.4	
	SN	212	4.05	75	45	4.3	136	36.4	0	1.4	
Exp. 2	CON	322	4.12	72	72	14.1	118	26.5	0	3.7	
	FA	322	4.19	49	49	79.3	37	9	0	3.5	
	SN	325	4.10	68	46	18.7	110	24.1	0	1.0	

CON, no additive; FA, formic acid 4 L t^{-1} fresh matter (FM); NaHe, sodium nitrite and hexamin
e mixture; LAB, lactic acid bacteria; NaHe0, sodium nitrite 900 g t^{-1} FM without hexamine; NaHe300, sodium nitrite 900 g t^{-1} FM with 300 g hexamine t^{-1} FM; NaHe600, sodium nitrite 900 g t^{-1} FM with 600 g hexamine t^{-1} FM; SN, sodium nitrite 900 g t^{-1} FM; DM, dry matter g kg^{-1}; Amm-N, ammonia-N g kg^{-1} N; Amm-N cor; ammonia-N, deducted all nitrogen applied through additive; g kg^{-1} DM: WSC, water soluble carbohydrates; LA, lactic acid; AA, acetic acid; BA, butyric acid; Eth, ethanol, Ethyl esters: sum of lactic acid ethyl esters and acetic acid ethyl esters.

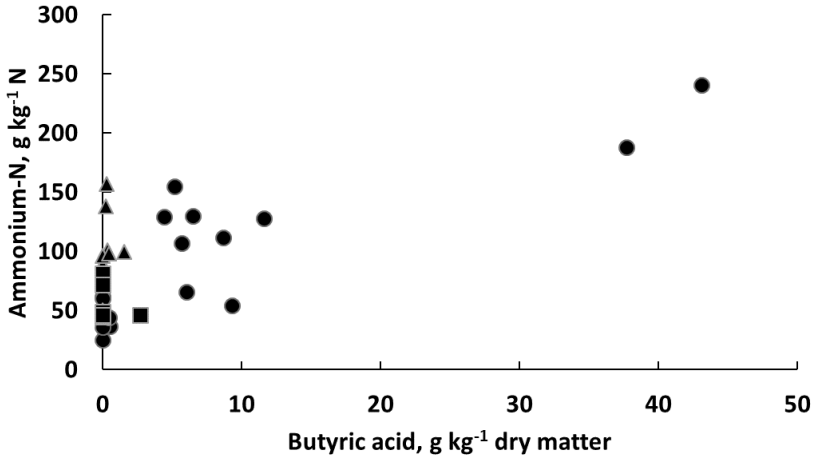

Figure 1 Relationship between ammonia-N (corrected by deducting all nitrogen applied through additive) and butyric acid concentrations of experimental silages in I (●), II (▲) and III (■).

The number of clostridial DNA-copies in the untreated control silages were at the same level (log 5.6) in I's four experiments. No DNA-copies were detected in II's experiments. The highest amounts of clostridia DNA-copies were found in III, possibly due to the high contamination of the herbage. Proteolytic clostridia were detected in high numbers (log 6.06 to log 8.12) only in III. The sum of clostridia DNA-copies in III's experiments was log 10.4.

Formic acid as silage additive

Formic acid treatment prevented butyric acid formation only in II (sub-experiment 1) and in III (sub-experiment 2). All other FA silages exposed butyric acid concentrations between 1.57 and 11.6 g kg^{-1} DM. According to Spiekers (2011), butyric acid values of quality silage are less than 3 g kg^{-1} DM. This limit value was exceeded in all FA silages in I.

The WSC concentrations of FA silages ranged from 33.6 to 208 g kg^{-1} DM, being higher in some silages than those in the pre-ensiled crop. High WSC values fit the fermentation profile of FA. The direct acidification of the herbage suppresses fermentation and catalyzes the hydrolyzation of plant cell wall degradation (McDonald et al., 1991, Hayashi et al. 2005), resulting in high residual WSC concentrations and low lactic acid concentration. The acidification level in experiments 3 and 4 of I did not prevent elevated ammonia-N concentrations in the FA silages. The utilized dose of 4 L pure FA per t FM did not decrease the pH enough to prevent butyric acid fermentation and resulted in butyric acid concentrations from 8.7 to 11.6 g kg^{-1} DM (I) when

pre-ensiled crop nitrate concentration was below 0.2 g kg^{-1} DM. Although FA is strongest aliphatic carbon acid, FA alone is unable to sufficiently reduce silage pH like mineral acids (AIV process) if the buffering capacity of the crop is high. The lower pH values compared with FA silage were observed when LAB was utilized as a silage additive (I).

When FA is used to support lactic acid fermentation, the application rate should not limit LAB proliferation, because lactic acid contributes to the final pH value. Clostridia tolerate the same pH-values as lactic acid bacteria; thus, the right dosing of FA is complicated. The effect of FA on decreasing silage pH in the initial phases of ensiling was investigated in II. FA decreased the pH instantly to 3.5 when the bi-crop was not wilted, resulting in butyric acid-free silage. The initial pH was 3.7 in the wilted bi-crop but increased very quickly to 4.3, resulting in butyric acid fermentation. The raising of the pH might be a result of the adjusting equilibrium in the crop between FA and the crop solutes (anions of plant acids, amino acids, ammonia).

Chamberlain and Quig (1987) obtained good silage quality with the application of 2 and 6 litres FA per tonne of ryegrass, but the fermentation quality was poor with 4 litres. On the other hand, both application rates, 4 and 6 l FA per tonne, produced good quality grass silage in Jaakkola et al.'s (2006) experiment. Finding the right dosing level is problematic due to variations in forage crop ensilability traits. Silages of low nitrate concentration need more FA to restrict butyric acid fermentation. All silages, including the untreated control, were free of butyric acid in Jaakkola et al.'s (2006) trial, suggesting sufficient nitrate concentration of grass. The use of FA in II and III resulted in low butyrate concentrations in low DM silages with nitrate concentrations higher than 3.8 g kg^{-1} DM in pre-ensiled crop (Figure 2).

A high number of clostridial DNA copies were found in FA silages in the experiments of I and III but not in II. The DNA-copy numbers in I varied from log 4.02 to log 5.90 g^{-1} FM. Both experiments of III contained log 11 DNA copies g^{-1} FM. All four investigated clostridia strains in III were found in high DNA-copy numbers. *C. tyrobutyricum* was the most abundant species.

Sodium nitrite as silage additive

Sodium nitrite was used along with hexamine in I, with different hexamine application rates in II, and as a sole additive in III. A mixture of SN and hexamine and SN alone prevented butyric acid fermentation and promoted lactic acid fermentation throughout all experiments. Butyric acid-free silages were obtained regardless of the pH of the silages when using SN.

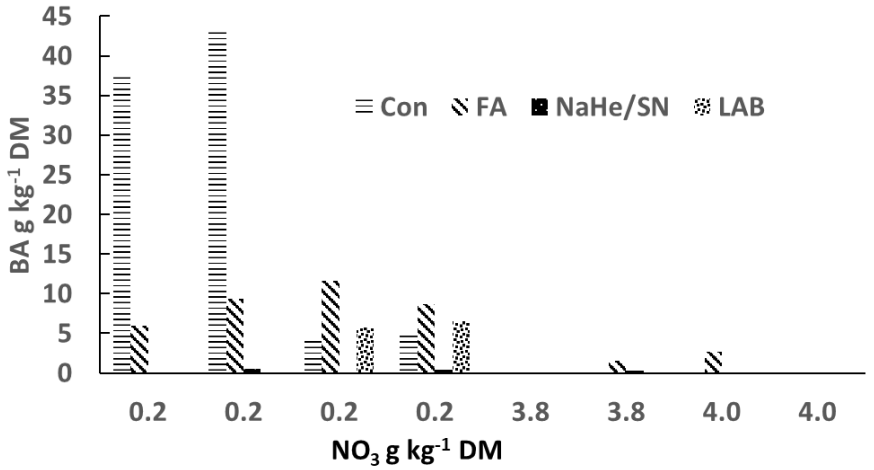

Figure 2 Relationship between nitrate (NO_3) concentration of pre-ensiled crops (publication I, 0.2; II 3.8 and III 4.0 g kg^{-1} dry matter) and butyric acid (BA) concentration of silages treated with different additives (CON, untreated control; FA, formic acid 4 L t^{-1}; NaHe, sodium nitrate+hexamine (I) and SN, sodium nitrite (II and III)).

Different hexamine application rates were investigated in II, because hexamine releases formaldehyde. Formaldehyde is forbidden as a preservation additive in foods because of toxicologic concerns (Lück and Jager, 1995). Increasing hexamine concentration had a decreasing effect on lactic acid fermentation, resulting in lower lactic acid concentrations and higher pH values. The ensiling results of NaHe (II) suggest that the utilization of hexamine as a silage additive in a mixture with sodium nitrite could be neglected. The SN results (III) support the neglection of hexamine as silage additive. The use of sodium nitrite without hexamine was reported by Knicky (2005), Knicky and Spörndly (2009) and Knicky and Spörndly (2011). The use of sodium nitrite along with different mixtures of sodium benzoate, potassium sorbate and sodium propionate resulted in butyric acid-free silages.

The clostridial contamination of NaHe silages was low in I (from log 2.46 to log 3.67 copies g^{-1} FM), and *C. tyrobutyricum* was the most abundant clostridia species. The contamination of SN silage with clostridia in III was similar to CON and FA silages.

Lactic acid bacteria

Good fermentation results were obtained with homofermentative lactic acid bacteria at the early maturity stage (I, exp.1 and 2) of white lupin-wheat bi-crop, while poor fermentation quality was exposed (I) at the late maturity stage

(I, exp. 3 and 4). The WSC concentration of the crop used at late maturity stage was only half compared with the WSC concentration at early maturity stage. This shows the dependence of homofermentative LAB on WSC. According to Weissbach et al. (1974), the ratio of WSC/BC should be more than 2.5 to obtain butyric acid-free silages. The WSC/BC ratio was below 1.5 in exp. 3 and 4 and higher than 2.5 in exp. 1 and 2. Thus, the LAB fermentation results of I confirm the findings of Weissbach et al. (1974), although the crop nitrate concentration was low.

The clostridial contamination of LAB silages was low when ensiled at the early maturity stage of a crop (log 2 copies g^{-1} FM) and high at the late maturity stage (log 6.41 copies g^{-1} FM).

The effect of the additives on volatile organic compounds

Volatile organic compounds like ethanol, ethyl acetate and ethyl lactate were measured to explore the formation of ethyl esters and the effect of additive treatment on the amount of ethyl esters (I and II). According to Weiss et al. (2016), the formation of ethyl esters correlates with the amount of ethanol. This correlation was found only in II. Formic acid- and sodium nitrite-treated silages had the lowest concentration of total ethyl esters.

6 CONCLUSIONS

1) White lupin-wheat bi-crop can be regarded as difficult to ensile due to its ensilability traits, which are affected by crop maturity, WSC concentration, white lupin to wheat ratio, buffering capacity, and nitrate concentration.
2) Additives were able to improve legume silage quality compared to untreated control silage in most of the experiments. The effect of additives on clostridial fermentation was influenced by the pre-ensiled nitrate concentration of the crop. Silage butyric acid concentration was high in experiments in which nitrate concentration of the crop was low. Increasing crop nitrate concentration supports the additive effect on clostridia.
3) The lactic acid bacteria-based additive was effective when herbage WSC concentration was sufficient, resulting in WSC/BC-ratio higher than 2.5. This confirms that lactic acid bacteria-based additive is less efficient than chemical additives when herbage WSC concentration is low.
4) Hexamine addition to sodium nitrite solution did not improve silage quality under trial conditions, suggesting that the use of hexamine as an additive produces no additional benefits.
5) Formic acid treatment at the rate of 4 L t^{-1} crop FM was less effective than nitrite-based additives in preventing clostridial fermentation in all silages of I, in wilted silages in II, and in unwilted silages of III. The complete failure of formic acid as a silage additive in white lupin-wheat silages might be connected to low nitrate concentration of the herbages in I and insufficient application rate of formic acid. Finding the right application rate for formic acid is crucial for ensiling success.
6) Clostridial fermentation in silage was suppressed efficiently by mixtures of sodium nitrite and hexamine and by sodium nitrite alone with no or only traces of butyric acid in all experiments. Thus, nitrite-based additives were more suitable than formic acid when ensiling legume-based forages prone to clostridial contamination.
7) Additive treatment did not influence aerobic stability. All silages were aerobically stable, indicating that stability of legume silages was not depending on the fermentation quality of silage.

7 FUTURE RESEARCH

The steady growth of agricultural farms and the further development of silage management place new demands on the ensiling of forage plants. The utilization of feed mixers and other automated feeding systems requires silages that are both anaerobically and aerobically stable. Silage heating during feeding and feed-out must be avoided due to the high losses of dry matter. A nitrogen-restrictive agricultural policy of the European Union could also lower the nitrate concentration of forage plants.

Sufficient nitrate concentration in the forage plants is important for the production of silage free from butyric acid. Thus, the nitrate concentrations of different forage crops grown under different conditions would be useful to study in more detail.

Combination preparations from homolactic lactobacilli with an additive against silage heating, e.g., sodium benzoate, are already on the market but could be investigated further and compared with heterolactic lactic acid bacteria. Mixtures of the salts against silage heating with an external proton donor like citric acid could be subject to future research.

8 REFERENCES

Atkins, C. A., Pate, J. S. & Sharkey, P. J. 1975. Asparagine metabolism – key to the nitrogen nutrition of developing legume seeds. Plant Physiology 56: 807–812.

Bergen, W. G., Byrem, T. M. & Grant, A. L. 1991. Ensiling characteristics of whole-crop small grains harvested at milk and dough stages. Journal of Animal Science 69: 1766–1774.

Borreani, G., Chion, A. R., Colombini, S., Odoardi, M., Paoletti, R. & Tabacco, E. 2009. Fermentative profiles of field pea *(Pisum sativum*), faba bean (*Vicia faba*) and white lupin (*Lupinus albus*) silages as affected by wilting and inoculation. Animal Feed Science and Technology 151: 316–323.

Chamberlain D.G. & Quig J. 1987. The effects of the rate of addition of formic acid and sulphuric acid on the ensilage of perennial ryegrass in laboratory silos. Journal of the Science of Food and Agriculture 38: 217–228.

Cremonesi, P., Vanoni, L., Silvetti, T., Morandi, S., & Brasca, M. 2012. Identification of *Clostridium beijerinckii, Cl. butyricum, Cl. sporogenes, Cl. tyrobutyricum* isolated from silage, raw milk and hard cheese by a multiplex PCR assay. Journal of Dairy Research 79: 319–323.

Dewhurst, R. J., Fisher, W. J., Tweed, J. K. S. & Wilkins, R. J. 2003. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. Journal of Dairy Science 86: 2598–2611.

Driehuis, F. & Van Wikselaar, P. G. 1996. The occurrence of alcoholic fermentation in high dry matter grass silages. Proceedings of the XIth International Silage Conference, University of Wales, Aberystwyth, p. 254-255.

Driehuis, F. & Oude Elferink, S.J.W.H. 2000. The impact of the quality of silage on animal health and food safety: A review. Veterinary Quarterly, 22:4, 212-216, DOI:10.1080/01652176.2000.9695061

Driehuis, F. 2013. Silage and the safety and quality of dairy foods: a review. Agricultural and Food Science 22: 16-34.

EFSA, 2006. Opinion of the scientific panel on additives and products or substances used in animal feed for the establishment of guidelines on the assessment of safety and efficacy of silage additives, on a request from the Commission under Article 7(5) of Regulation (EC) No 1831/2003. The EFSA Journal 349: 1–10. https://doi.org/10.2903/j.efsa.2006.349

Ercolani, G. L. 1997. Note: Occurrence and persistence of culturable clostridial spores on the leaves of horticultural plants. Journal of Applied Microbiology 82: 137-140.

Fiebig, H.-J., Rinno, G., & Ebert, K. 1974. Silierfähigkeit und Futterwert von Gräsern in Abhängigkeit von Stickstoffdüngung und Schnittzeitpunkt, 1. Mitt.: Wasserlösliche Kohlenhydrate, Rohprotein und Silierfähigkeit. Archiv für Acker- und Pflanzenbau u. Bodenkunde 18: 293-303.

Fraser, M. D., Fychan, R., & Jones, R. 2005. The effect of harvest date and inoculation on the yield and fermentation characteristics of two varieties of white lupin (*Lupinus albus*) when ensiled as a whole-crop. Animal Feed Science and Technology 119: 307–322.

Hayashi, T., Yoshida, K., Park, Y. W., Konishi, T. & Baba, K. 2005. Cellulose metabolism in plants. International Review of Cytology 247: 1-34.

Hellberg A. 1967. A combination of nitrite and hexamine as an additive in the ensiling of herbage. Journal of the British Grassland Society 22: 289-292.

Henderson, A. R., McDonald, P. & Woolford, M. K. 1972. Chemical changes and losses during ensilage of wilted grass treated with formic acid. Journal of the Science of Food and Agriculture 23: 1079-1083.

Hoedtke, S. 2007. Die Quantifizierung der Osmolalität in Futterpflanzenund ihre Veränderung in verschiedenen Stadien der Silierung. Dissertation. Institut für Nutztierwissenschaften und Technologie der Agrar- und Umweltwissenschaftlichen Fakultät der Universität Rostock. Institut für Grünland- und Futterpflanzenforschung.161 p.

Huhtanen, P., Jaakkola, S. & Nousiainen, J. 2012. An overview of silage research in Finland: from ensiling innovation to advances in dairy cow feeding. Proceedings of the XVI International Silage Conference Hämeenlinna, Finland, 2-4 July 2012. p. 16-33.

Jaakkola, S., Kaunisto, V. & Huhtanen, P. 2006. Volatile fatty acid proportions and microbial protein synthesis in the rumen of cattle receiving grass silage ensiled with different rates of formic acids. Grass and Forage Science 61: 282-292.

Jaakkola, S., Saarisalo, E. & Heikkilä, T. 2009. Formic acid treated whole crop barley and wheat silages in dairy cow diets: Effects of crop maturity proportion in the diet and level and type of concentrate supplementation. Agricultural and Food Science 18: 234–256.

Jones, R., Fychan, R., Evans, S. & Roberts, J. 1999. Effects of wilting and application of bacterial inoculant on the fermentation characteristics of lupin silage. In T. Pauly (Ed.), Silage production in relation to animal performance, animal health, meat and milk quality (pp. 98–99). Conference Proceedings of the XIIth International Silage Conference. July 5–7, 1999, Uppsala, Sweden.

Jonsson, A. 1989. The role of yeasts and Clostridia in Silage deterioration. Rapport- Sveriges Lantbruksuniversitet, Institutionen för Mikrobiologi, no 42.

Jänicke, H. 2011. Grobfutter- und Substraterzeugung. In: Praxishandbuch Futter- und Substratkonservierung. 8 Auflage, DLG-Verlag, Frankfurt/M., 2012, S. 27.

Kaiser, E. & Weiss, K. 1997. Zum Gärungsverlauf bei der Silierung von nitratarmem Grünfutter 2. Mitteilung: Gärungsverlauf bei Zusatz von Nitrat, Nitrit, Milchsäurebakterien und Ameisensäure. [On the fermentation process during ensiling of green forage low in nitrate. 2. Fermentation course with the addition of nitrate, nitrite, lactic acid bacteria and formic acid]. Archiv für Tierenährung 50: 187–200. https://doi.org/10.1080/17450399709386130

Kaiser, E. & Weiss, K. 2007. Nitratgehalte im Grünfutter- bedeutung für Gärqualität und siliertechnische Massnahmen. Übersichten zur Tierernährung 35: 13-30.

Keith, S. M., MacFarlane, G. T. & Herbert, R. A. 1982. Dissimilatory nitrate reduction by a strain of *Clostridium butyricum* isolated from estuarine sediments. Archives of Microbiology 132: 63–66.

Kleinschmidt, D. H. & Kung Jr, L. 2006. A Meta-Analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. Journal of Dairy Science 89: 4003-4013.

Knický, M. 2005. Possibilities to Improve Silage Conservation Effects of Crop, Ensiling Technology and Additives. Faculty of Veterinary Medicine and Animal Science Department of Animal Nutrition and Management Uppsala Doctoral thesis Swedish University of Agricultural Sciences Uppsala.

Knický, M. & Spörndly, R. 2009. Sodium benzoate, potassium sorbate and sodium nitrite as silage additives. Journal of the Science of Food and Agriculture 89: 2659–2667.

Knický, M. & Spörndly, R. 2011. The ensiling capability of a mixture sodium benzoate, potassium sorbate, and sodium nitrite. Journal of Dairy Science 94: 824-831.

Koeppen, B.M. & Stanton, B.A. 2019. Renal Physiology 6th edition. Elsevier Inc. p. 2-5.

Kung, L. Jr., Strokes, M. R. & Lin, C.J. 2003. Silage Additives. In: Buxton, D.R., Muck, R.E. & Harrison, J.H. (eds.). Silage Science and Technology. American Society of Agronomy, Inc. p. 305-360.

Lambert, R. J. & Stratford, M. 1999. Weak-acid preservatives: Modelling microbial inhibition and response. Journal of Applied Microbiology 86: 157–164. Landwirtschaft Braunschweig-Völkenrode (FAL) Braunschweig, Germany.

Lingvall, P. & Lättemäe, P. 1999. Influence of hexamine and sodium nitrite in combination with sodium benzoate and sodium propionate on fermentation and hygienic quality of wilted and long cut grass silage. Journal of the Science of Food and Agriculture 79: 257–264.

Lättemäe, P. & Lingvall, P. 1996. Influence of hexamine and sodium nitrite in combination with sodium benzoate and sodium propionate on fermentation and storage stability of wilted and long cut grass silage. Swedish Journal of Agricultural Research 26: 135-146.

Lück, E. & Jager, M. 1995. Chemische Lebensmittelkonservierung (Chemical food preservation), 3rd edn. Springer-Verlag Berlin, Heidelberg, GmbH. 273 p.

McDonald, P. & Henderson, A. R. 1962. Buffering capacity of herbage samples as a factor in ensilage. Journal of the Science of Food and Agriculture 13: 395–400.

McDonald, P., Henderson, A. R. & Heron, S. J. E. 1991. The Biochemistry of Silage 2nd edition. Chalcombe Publications, Marlow, UK. 340 p.

Mengel, K. 1991. Ernährung und Stoffwechsel der Pflanze. 7. Auflage, Verl.Gustav Fischer, Stuttgart

Pahlow, G., Rammer, C., Slottnerand, D. & Tuori, M. 2002. Ensiling of legumes. Landbauforschung Voelkenrode, Sonderheft 234.

Pahlow, G., Muck, R., Driehus, F., Oude Elferink, S.J.W.H. & Spoelstra, S.F. 2003. Microbiology of Ensiling. In: Buxton, D.R., Muck, R.E. & Harrison, J.H. (eds.). Silage Science and Technology. American Society of Agronomy, Inc. p. 31–93.

Playne, M.J. & McDonald, P. 1966. The buffering constituents of herbage and silage. Journal of the Science of Food and Agriculture 17: 264-268.

Pettersson, K. 1988. Ensiling of forages. Factors affecting silage fermentation and quality. Dissertation. Swedish University of Agricultural Sciences. Department of Animal Nutrition and Management. Report 179.

Pursiainen, P. & Tuori, M. 2008. Effect of ensiling field bean, field pea and common vetch in different proportions with whole-crop wheat using formic acid or an inoculant on fermentation characteristics. Grass and Forage Science 63: 60-78.

Podkowka, W. 1969. Bewertung und Klassifizierung von Futterpflanzen im Hinblick auf die Gärfähigkeit. Proc. 3rd Gen. Meeting Europ. Grassl. Fed., Braunschweig, 41-52.

Rammer, C. 1996. Quality of grass silage infected with spores of Clostridium tyrobutyricum. Grass and Forage Science 51: 88-95.

Rojas, E.R. & Huang, K.C. 2018. Regulation of microbial growth by turgor pressure. Current Opinion in Microbiology 42: 62-70.

Schmidt, L., Weissbach, F., Wernecke, K.D. & Hein, E. 1971. Erarbeitung von Parametern für die Vorhersage und Steuerung des Gärverlaufes bei der Grünfuttersilierung. Bericht des Oskar Kellner-Institutes, Rostock.

Spiekers, H. 2011. Ziele in der Wiederkäuerfütterung. In: Praxishandbuch Futter- und Substratkonservierung. 8 Auflage, DLG-Verlag, Frankfurt/M., 2012, S. 13.

Spoelstra, S. F. 1983. Inhibition of clostridial growth by nitrate during the early phase of silage fermentation. Journal of the Science of Food and Agriculture 34: 145-152.

Spoelstra, S.F. 1985. Nitrate in silage. Grass and Forage Science 40: 1–11. https://doi.org/10.1111/j.1365-2494.1985.tb01714.x

Spolders, M. 2006. Anorganische Stoffe / Kontaminanten Nitrit/Nitrat. Völkenrode Sonderheft 294: 56–60 Landbauforschung. http://literatur.thuenen.de/digbib_extern/bitv/zi040833.pdf.

Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. K. & Schmidt, T. M. 2015. rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucleic Acids Research 43: D593–D598.

Virtanen, A. I., 1933. The A.I.V. method for the preservation of fresh fodder. Acta Chemica Fennica A 6: 13-28.

Vissers, M. M. M., Driehuis, F., Te Giffel, M. C., De Jong, P. & Lankveld, J. M. G. 2007. Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration. Journal of Dairy Science 90: 928–936.

Weiss, K. 2001. Gärungsverlauf und Gärqualität von Silagen aus nitratarmem Grünfutter. [Fermentation process and fermentation quality of silage from green forage low in nitrate]. Dissertation. Humboldt Universität. 181 p.

Weiss, K., Kroschewski, B. & Auerbach, H. 2016. Effects of air exposure, temperature and additives on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in corn silage. Journal of Dairy Science 99: 8053–8069.

Weissbach, F. 1968. Beziehungen zwischen Ausgangsmaterial und Gärungsverlauf bei der Grünfuttersilierung [Relations between the herbage and the course of fermentation in ensiling green fodder]. Habilitation. University of Rostock, Germany.

Weissbach, F. 1992. (Bestimmung der Pufferkapazität). Determination of buffering capacity of silage crops. Manual, Bundesforschungsanstalt für Landwirtschaft Braunschweig-Völkenrode (FAL), Institut für Grünland- und Futterpflanzenforschung. 3 p.

Weissbach, F. 1999. Consequences of grassland de-intensification for ensilability and feeding value of herbage. Landbauforschung Völkenrode (FAL). Contributions of Grassland and Forage research to the Development of Systems of Sustainable Land Use, pp. 41-53.

Weissbach, F., Schmidt, L. & Hein, E. 1974. Method of anticipation of the run of fermentation in silage making based on the chemical composition of

the green fodder. In: Proceedings of the 12th International Grassland Congress. Moscow 1974. Russian Academy of Agricultural Sciences, Lugovaya, p.663-673.
https://www.openagrar.de/receive/openagrar_mods_00027701

Weissbach, F. & Haacker, K. 1988. Über die Ursachen der Buttersäuregärung in Silagen aus Getreidepflanzen. Das wirtschaftseigene Futter 34: 88-99

Wieringa, G. W. 1958. The effect of wilting on butyric acid fermentation in silage. Netherlands Journal of Agricultural Science 6: 204-210.

Wieringa, G. W. & Beck, T. 1964. Investigations on the use of cultures of lactic acid bacteria in the preparation of silage in small containers. Obtaining active Lactobacillus cultures for inoculation trials. Das wirtschaftseigene Futter 10: 34-44.

Wilkins, R. 1983. The preservation of forage. In: Ørskov, E. R. (ed) Feed Science, pp. 231-255. Elsevier. Amsterdam.

Wilman, D. 1980. Early spring and late autumn response to applied nitrogen in four grasses. 1. Yield, number of tillers and chemical composition. The Journal of Agricultural Science-Cambridge Core 94: 425-442.

Woolford, M.K. 1975. Microbiological screening of food preservatives, cold sterilants and specific antimicrobial agents as potential silage additives. Journal of the Science of Food and Agriculture 26: 229-237

Yingxi, G. 2016. The Effect of Inhibitors on Butyric Acid Fermentation by *Clostridium tyrobutyricum*. North Carolina State University. Raleigh, North Carolina. Thesis. 131 p.