Impact of hexamine addition to a nitrite-based additive on fermentation quality, Clostridia and Saccharomyces cerevisiae in a white lupin-wheat silage

König, Walter

2019-03-15

König , W , Konig , 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 , vol. 99 , no. 4 , pp. 1492-1500 . https://doi.org/10.1002/jsfa.9322

http://hdl.handle.net/10138/324194
https://doi.org/10.1002/jsfa.9322

acceptedVersion

Impact of hexamine addition to a nitrite based additive on fermentation quality, clostridia and *Saccharomyces cerevisiae* in a white lupin-wheat silage

W. König[a*], E. König[a], K. Weiss[b], T.T. Tuomivirta[c], H. Fritze[c], K. Elo[a], A.Vanhatalo[a] and S. Jaakkola[a]

[*] Correspondence to: W. König, Department of Agricultural Sciences, P.O. Box 28, FI-00014 University of Helsinki, Finland, walter.konig@helsinki.fi

[a]Department of Agricultural Sciences, P.O. Box 28, FI-00014 University of Helsinki, Finland, [b]Humboldt Universität zu Berlin, Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Science, Invalidenstraße 42, 10115 Berlin

[c]Natural Resources Institute Finland, Latokartanonkaari 9, FI-00790 Helsinki

Abstract

BACKGROUND: Nitrite and hexamine are utilised as silage additives because of their adverse effect on clostridia and clostridia spores. The effect of sodium nitrite and sodium nitrite/hexamine mixtures on silage quality was investigated. A white lupin-wheat mixture was treated with sodium nitrite (NaHe0) (900 g t^{-1} forage), or mixtures of sodium nitrite (900 g t^{-1}) and hexamine. The application rate of hexamine was 300 g t^{-1} (NaHe300) or 600 g t^{-1} (NaHe600). Additional treatments were the untreated control (Con), and formic acid (FA) applied at a rate of 4 L t^{-1}.

RESULTS: Additives improved silage quality noticeably only by reducing silage ammonia content compared with the control. The addition of hexamine to a sodium nitrite solution did not improve silage quality compared with the sole sodium nitrite solution. The increasing addition of hexamine resulted in linearly rising pH values ($P<0.001$) and decreasing amounts of lactic acid ($P<0.001$). Sodium nitrate based additives were more effective than formic acid

to butyric acid formation. Additives did not restrict the growth of *Saccharomyces cerevisiae* compared to the control.

CONCLUSION: The addition of hexamine did not improve silage quality compared with a solution of sodium nitrite.

Keywords: clostridia, hexamine, sodium nitrite, qPCR, silage, white lupin

Introduction

Nitrate and nitrite are utilised as silage additives because of their adverse effect on clostridia and clostridia spores.[1] In addition to additives, nitrate found naturally in forage crops affects silage fermentation. Nitrate in fresh forage is reduced to several nitrogen compounds during silage fermentation. Immediately after ensiling the number of nitrate reducing enterobacteria increases and nitrite is accumulating.[2] Wieringa[3] ensiled grasses ranging in nitrate concentration from 1 – 20 g kg^{-1} dry matter (DM). His study revealed that 4 – 8 g kg^{-1} DM nitrate in forage resulted in butyric acid free silage, but silage containing both lower and higher nitrate levels were prone to butyric acid fermentation. Weiss[4] found that a nitrate concentration of pre-ensiled forage below 4.43 g NO_3 kg^{-1} DM exposed a higher risk for butyric acid formation in grass and grass-legume silages when ensiled without silage additive.

Lupines diversify crop rotation and choice of legume plant species being an alternative for faba bean and peas even in a boreal climate[5]. Especially white lupin (*Lupinus albus* L.) is a potential legume to be used as whole crop silage because of its high yield[6]. However, legumes are considered as difficult to ensile due to their low DM content, high buffering capacity[7] and low nitrate content[8]. A former study of König *et al.*[9] revealed that a mixture of sodium nitrite and hexamine was most effective to inhibit butyric acid fermentation and clostridia when different

mixtures of white lupine-wheat bi-crops low in nitrate content were ensiled. The administered formic acid application rate (4 L t^{-1} fresh matter (FM), 1000 g kg^{-1} formic acid) was insufficient to decrease pH enough for preventing the growth of clostridia and butyric acid fermentation. More information is needed to control ensiling process of white lupin and other legumes difficult to ensile.

The use of formic acid as additive has exposed inconsistent anti-clostridial effects on the fermentation quality of silages[10]. This may be partly related to lysis of plant cells caused by formic acid. Rammer[11] suggested that cell sap provides saccharolytic clostridia with nutrients and enhances clostridia growth. He infected grass with spores of *Clostridium tyrobutyricum* and found in silage no anti-clostridial effect of formic acid (850 g kg^{-1}) applied at a rate of 4 L t^{-1} herbage.

Hellberg[12] started to investigate mixtures of nitrite and hexamine which are still used in commercial products. However, there are concerns about the effects of hexamine on human health.[13] From this point of view it is important to investigate the effect of hexamine on silage quality. Hexamine itself has no anti-microbial effect.[1] The anti-microbial effect is based on formaldehyde which is released under acidic environmental conditions from hexamine[1]. Formaldehyde reacts with proteins and impairs enzymes of the micro-organism. Since the effect of formaldehyde is not specific to microbes, plants with high protein content may reduce the efficacy of formaldehyde.[1] Utilizing formaldehyde as silage additive is well investigated.[12,14,15,16] Trying to improve the effect of formaldehyde on silage fermentation quality, different mixtures of formaldehyde and other additives were investigated. One of them was a mixture of hexamine and sodium nitrite. Investigations of Hellberg[12] revealed that silage quality of nitrite treated forages were in most cases superior to those treated with hexamine alone. However, some of those trials showed a synergetic effect of hexamine and sodium nitrite.

Formaldehyde impairs lactic acid bacteria growth and induces an increasing pH[12]. On the other hand, certain soil indigenous bacteria and yeasts utilize hexamine as a sole source of carbon, nitrogen and energy.[17] Based on that, increasing levels of hexamine in silage might increase simultaneously yeast fermentation. More information is needed on the effects of increasing amounts of hexamine in mixtures with sodium nitrite on silage quality.

Two experiments were conducted to study the effects of sodium nitrite and sodium nitrite-hexamine mixtures on the quality of unwilted and wilted white lupine-wheat silage compared with formic acid treated and untreated silage. The main target of the study was to investigate if the efficiency of sodium nitrite based additive is improved by increasing the amount of hexamine in the additive. It was hypothesized that i) the use of additives prevents clostridial and yeast fermentation; ii) formic acid is less effective in preventing clostridia and *Saccharomyces cerevisiae* in silages than sodium nitrate or mixtures of sodium nitrite and hexamine, iii) adding increasing amount of hexamine suppresses clostridia and *S. cerevisiae* proliferation in silage.

Materials and methods

Treatments and silage preparation

White lupin (*Lupinus albus,* variety Feodora, 200 kg/ha) and spring wheat (*Triticum aestivum L.*, variety Amaretto, 80 kg/ha) were sown as a mixture on 19 May 2014 at the Viikki Research Farm of University of Helsinki, Finland (60^0N, 25^0E). The experimental field area was fertilized in the previous autumn with livestock manure and in spring with a nitrogen fertilizer resulting in total 50 kg N ha^{-1}.

The bi-crop was used for two separate ensiling experiments. For the experiment 1, the bi-crop was harvested and ensiled unwilted on 19 August, and for the experiment 2 it was cut on 16 August and ensiled after 40 h wilting time. The bi-crop was harvested at a stubble height of about 10 cm utilizing a disc mower (Krone EasyCut 3210 CV, Maschinenfabrik Bernard Krone GmbH, Spelle, Germany). At that time, wheat was at the end of the dough stage and lupine pods were filled to 75% with the green seeds. The development stage of white lupine was 4.3 according to the scale of Dracup and Kirby[18]. Representative samples were collected from the experimental field area for botanical analyses before harvesting the bi-crop. The samples were taken from six randomly chosen areas of 0.25 m^2 of size.

The forages were chopped using a laboratory chopper (Wintersteigner®, Ried im Innkreis, Austria) to give a chop length of 1-4 cm. After chopping, forage was treated with the following additives: 4.2 L t^{-1} formic acid (FA; Sigma Aldrich, St. Louis, USA; 950 g kg^{-1}) which equals 4 L pure formic acid (1000 g kg^{-1}) per ton fresh matter (FM) of forage and three mixtures of sodium nitrite (Sigma Aldrich, St. Louis, USA) and hexamine (Sigma Aldrich, St. Louis, USA) (NaHe). The application rates of hexamine were 0 g t^{-1} (NaHe0), 300 g t^{-1} (NaHe300) and 600 g t^{-1} (NaHe600) supplemented with a constant rate of 900 g t^{-1} of sodium nitrite (Table 1). The control was treated with 10 mL tap water per kg FM and the additives were applied as a water solution with10 mL kg^{-1} FM including additive and water. The additive was applied from a spray bottle to the forage batch for each treatment and thoroughly mixed during application. After additive treatment, forage samples were taken for immediate pH determination. The forage was ensiled in 1.5 L glass silos (Weck®, Wher-Oflingen, Germany) with three replicates per treatment. The fermentation gases were allowed to leak through the rubber seal between glass silo and lid. The amount of forage filled in the silos was 1050 g (unwilted) and 900 g (wilted). The density of the unwilted and the wilted compacted forage in the silos was 105 and

144 kg DM per m^3, respectively. Silos were stored at an ambient room temperature (20–22ºC), and opened 154 days after ensiling.

The same forages were ensiled also in glass silos with a volume of 120 mL to study the effect of silage pH decrease at the early phase of ensiling. For each treatment, eight replicate silos were used. The amount of forage filled in the silos was 90 g of unwilted forage and 80 g of wilted forage, the density being 112 and 160 kg DM per m^3, respectively. The silos were sealed with a rubber stopper and a screw cap. Two silos per treatment were opened 3 h, 6 h, 18 h and 168 h after treatment and silage pH was measured.

Chemical analysis and aerobic stability

A pre-ensiling sample of untreated bi-crop was taken for immediate DM and pH determination (SevenCompactTM S220 pH, Mettler-Toledo Ltd, Leicester, Great Britain) and for later analyses. Dry matter content was determined by drying the samples at 105ºC for 24 h in an oven (Memmert, Memmert GmbH, Schwabach, Germany). Fresh samples were frozen (-20ºC) for analyses of buffering capacity (BC), total and soluble nitrogen, water soluble carbohydrates (WSC), nitrate and clostridia. For analyses of ash, starch, neutral detergent fibre (NDF) and *in vitro* digestibility samples were dried at 60ºC for 48 hours in a ventilated drying chamber (Memmert, Memmert GmbH, Schwabach, Germany) and after that they were ground through a 1-mm sieve using a laboratory mill (KT-3100, Koneteollisuus Oy, Helsinki, Finland).

After opening the 1.5 l volume silos, the content was mixed, and samples were taken for immediate DM, pH and aerobic stability analyses. Samples for fermentation quality and clostridia were stored at -20ºC for later analyses. The silage fermentation parameters lactic acid, WSC, volatile fatty acids (VFA), alcohols, acetone and ethyl esters were analysed at the Humboldt Universität zu Berlin, while pH, nitrogen, ammonia-N, aerobic stability and all

herbage chemical analyses were made at the University of Helsinki with the methods reported in detail by König *et al.*[9] Briefly, buffering capacity of fresh herbage was measured according to Weissbach[19]. The content of N was determined by Kjeldahl method[20], and the contents of herbage starch[21] and WSC[22,23] and the content of silage ammonia N[24] were analyzed by a colorimetric method. Neutral detergent fibre was measured using the method of van Soest *et al.*[25] with amylase treatment. The results were reported including residual ash. The measurement of the content of digestible organic matter in DM (DOMD) was based on *in vitro* pepsin-cellulase solubility[26] with the modifications of Nousiainen *et al.*[27] and the results were calculated according to Huhtanen *et al.*[28] Forage nitrate content was measured using the combined nitrate ion selective electrode (perfectION, Mettler-Toledo AG, Schwerzenbach, Switzerland) and the nitrate interference suppressor solution of the manufacturer. The samples were prepared for the measurement according to Bedwell *et al.*[29] Water-soluble carbohydrates of silages were analysed by using the antron method* and lactic acid by high performance liquid chromatography according to Weiss and Kaiser[30]. Volatile fatty acids (acetic, propionic, isobutyric, butyric, isovaleric, valeric, and caproic acid), and alcohols (methanol, ethanol, propanol) were assessed by gas chromatography (GC) according to Weiss[4] and esters (ethyl lactate and ethyl acetate) by GC according to Weiss and Sommer[31].

Silage oven dried DM content was corrected for volatile substances corresponding to Weissbach and Strubelt[32]: $DM_c = DM_n + (1.05 – 0.059 \times pH) \times FA + 0.08 \times LA + 0.77 \times PD + 0.87 \times BD + 1.00 \times AL$, where DM_c is the corrected DM, DM_n non-corrected DM, FA the sum of volatile fatty acids ($C_2 – C_6$), LA lactic acid, PD 1,2-propandiol, BD 2,3-butandiol and AL the sum of remaining alcohols ($C_1 – C_4$). Aerobic stability was measured over period of 12 days and expressed as time elapsed until the temperature rose 2°C over the ambient temperature.[33]

Clostridium and Saccharomyces cerevisiae analyses using qPCR

The qPCR analyses of 4 *Clostridium* species (*C. butyricum, C. tyrobutyricum, C. sporogenes* and *C. perfringens*) were conducted in the laboratory of Natural Resources Institute of Finland. For each DNA extraction two to three grams of pre-ensiling herbage or silage were weighed and samples were homogenized with ULTRA-TURRAX® TP-18/10 (Janke and Kunkel GmbH and Co KG IKA-Werk, Staufen, Germany) in 10 mL of NEN 6877 milk-lactic-acid-glucose medium. Homogenates were centrifuged using 10 000 g for 15 min at 23°C. Approximately 200 mg of pellet per sample was collected for DNA extraction. The DNA extraction was conducted using Macherey-Nagel NucleoSpin® Soil kit (Macherey-Nagel GmbH and Co. KG**,** Düren, Germany) by using SL1 lysis buffer without SX enhancer as described by the manufacturer. Detailed description of the methods used in *Clostridium* analyses is given by König *et al.*[9]

The DNA extraction from silage for *S. cerevisiae* analyses followed the protocol used for *Clostridium* species, except following step: 30 grams of silage was homogenized in 200 mL distilled water for DNA extraction.

The qPCR reactions for *S. cerevisiae* were dispensed to optical 384-wellplates (Roche Diagnostics GmbH, Mannheim, Germany) using EpMotion 5070 automated pipetting system (Eppendorf AG, Hamburg, Germany). Sample DNA (2.5 µL) and mixture (7.5 µL) composed of 2 × SYBR Green Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), primers (5 pmol /µL/each) and DNase/RNase free water were added into each well. Primer sequences were based on Hierro *et al.*[34]

LightCycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) was used in qPCR. Each DNA sample was run in quadruplicate. The temperature profile of the real-time

206 PCR was as follow: initial denaturation step for 5 min at 95 °C, followed by 45 amplification
207 cycles for 10 sec at 95 °C, 20 sec at 55 °C, and 30 sec at 72 °C. Seven standard dilutions (from
208 0.00016 ng uL^{-1} to 2.5 ng uL^{-1} were amplified always on the same plate as samples. Raw
209 amplification data from LightCycler 480 was analysed using LinRegPCR software.[21] Results
210 from qPCR were presented as copy numbers per gram of silage.
211
212 **Calculations and statistical analysis**
213 The fermentation coefficient (FC) for pre-ensiling crops and mixtures was calculated as FC =
214 DM (g kg^{-1})/10 + 8 x WSC (g kg^{-1} DM)/BC(g kg^{-1} DM).[35] The minimum DM content of ensiled
215 herbage (DM_{min}) needed to ensure high fermentation quality of silage was calculated using the
216 equation DM_{min} (g kg^{-1}) = 450 + 80 x WSC (g kg^{-1} DM)/BC(g kg^{-1} DM).[36] A corrected N content
217 was calculated for NaHe silages by deducting all nitrogen added with additive from the analysed
218 amount of total nitrogen and ammonia nitrogen.
219
220 The results for fermentation quality parameters and clostridial numbers were analysed
221 separately for the two trials. Normally distributed variables were analysed by ANOVA using
222 the Mixed procedure of SAS (SAS 9.3, Institute Inc., Cary, NC) with a model $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$,
223 where Y_{ij} is the observation, μ the overall mean, α_j the effect of treatment and ε_{ij} the error term.
224 Sums of squares for treatment effects were further separated into single degree of freedom
225 comparisons using orthogonal contrasts to assess: 1) efficacy of using additives (FA, sodium
226 nitrite and NaHe *vs.* control); 2) differences in the efficacy between chemicals (NaHe *vs.* FA)
227 3) linear effect of increasing application rate of hexamine and 4) quadratic effect of increasing
228 application rate of hexamine. The level of significance was set at $P<0.05$. Non-normal
229 distributed data were tested with the Kruskall-Wallis non-parametric test (SPSS, version 21,
230 IBM, Armonk, USA) and when significant, the differences between the treatments were
231 analysed by pairwise testing (Dunn-Bonferroni). Statistically significant differences ($p<0.05$)

between the treatments are expressed using different letters (a, b). The linear relationship between average ethanol and the sum of ethyl lactate and ethyl acetate contents, and between ethanol content and *S. cerevisiae* numbers in silages were calculated by using the REG procedure of SAS (SAS 9.3, Institute Inc., Cary, NC, USA).

Results

Herbage botanical and chemical composition

The proportions of white lupin, wheat and weeds in the bi-crop before harvesting were on fresh weight basis 0.70, 0.26 and 0.04, respectively. On DM basis, the respective values were 0.42, 0.51 and 0.07.

The chemical composition of the herbages prior to ensiling and the qPCR clostridia results are shown in Table 2. The DM content was 150 g kg^{-1} and 240 g kg^{-1} for the unwilted and wilted bi-crop, respectively. The content of WSC in DM basis was at the same level in both forages while in FM basis, it was 17.2 g kg^{-1} in the unwilted and 26.2 g kg^{-1} in the wilted bi-crop. The calculated FC was 29.6 in the unwilted and 39.6 in the wilted bi-crop. The nitrate content was the same for both wilted and unwilted bi-crop (3.8 g kg^{-1} DM).

Quantitative PCR analyses revealed the contamination of the forage with clostridia and *S. cerevisiae* (Table 2). The unwilted forage contained 5.3 log copies g^{-1} FM of *C. perfringens*, 2.3 log copies g^{-1} FM *C. butyricum* and 7.43 log copies g^{-1} FM *S. cerevisiae*. The wilted forage contained 9.6 log copies g^{-1} FM of *C. tyrobutyricum*, 2.6 log copies g^{-1} FM *C. butyricum* and 6.81 log copies g^{-1} FM *S. cerevisiae*.

Silage aerobic stability and fermentation quality

The effects of additives on fermentation quality of silages are presented in Tables 3 and 4. The additive treatments are compared within experiments, not between unwilted and wilted silages in experiments 1 and 2, respectively. All the 42 investigated silages were aerobically stable for the whole measurement period of 12 days and therefore no results are presented.

Additives versus untreated control

The average pH of all additive treated silages was higher than in untreated control silage both in the unwilted ($P<0.05$) and wilted ($P<0.01$) silages. Lactic acid and acetic acid contents were lower ($P<0.001$) in the treated silages than in control silage in both experiments, while the average WSC content of the treated silages in experiment 1 was higher compared with the control silage ($P<0.001$). Only in experiment 2 the uncorrected ammonia-N content was lower in treated silages than in control silage *(*$P<0.01$*)*. However, additive treatment decreased the proportion of corrected ammonia-N in in both experiments ($P<0.001$). The use of additives resulted in lower ethanol, ethyl lactate, and the sum of ethyl lactate and ethyl acetate content in all silages compared with untreated control silage ($P<0.001$).

Sodium nitrite–hexamine based additives versus formic acid

In experiment 1 and 2 the average pH, and contents of lactic acid and acetic acid of all NaHe treated silages were higher compared with FA silages ($P<0.001$). Butyric acid content of NaHe silages in experiment 2 was lower ($P<0.05$) compared with FA silage. In experiment 1 NaHe treated silages contained less residual WSC than FA treated silages ($P<0.001$).

Unwilted NaHe silages exposed higher ammonia-N and corrected ammonia-N values compared with FA treated silages ($P<0.001$) in experiment 1. In experiment 2 the uncorrected ammonia-N content of NaHe silages was higher ($P<0.001$) than that of FA silages. The methanol and ethanol values of NaHe silages in experiment 1 were higher than those of FA silages ($P<0.01$)

while in experiment 2 the content of methanol was higher (P<0.001) and ethanol lower in NaHe than FA silages (P<0.001). The amount of ethyl acetate in experiment 1 was higher (P<0.05) in NaHe0 and NaHe600 treated silage than in FA silage and lower (P<0.01) in all NaHe treated silages than in FA silages in experiment 2. The content of ethyl lactate and the sum of ethyl lactate and ethyl acetate in NaHe silages were higher than in FA silage in experiment 1 (P<0.001) and lower in experiment 2 (P<0.001).

Addition of increasing amounts of hexamine to a sodium nitrite solution

In both experiments the addition of hexamine raised linearly silage pH (P<0.001) and decreased lactic acid content (P<0.05*).* Acetic acid values linearly increased with increasing amounts of hexamine in experiment 1 (P<0.05) but decreased in experiment 2 (P<0.01). The content of WSC increased between NaHe0 and NaHe600 in wilted silage (P<0.05). In both experiments silage uncorrected ammonia-N proportion grew linearly with increasing hexamine application rate (P<0.05).

Increasing rate of hexamine accumulated linearly the amount of methanol in silage in both experiments (P<0.001), while ethanol and the sum of ethyl lactate and ethyl acetate accrued curvilinearly only in experiment 1 (P<0.05). Silage ethyl lactate content increased linearly in experiment 1 (P<0.05) and decreased (P<0.01) with increasing amounts of hexamine in experiment 2. A strong linear relationship was found between silage ethanol and total ester amounts (Figure 3).

Effect of additive treatment* on *pH at initial phase of ensiling

In both experiments 1 and 2, the pH started to fall from an initial level of herbage pH 6.28 and pH 6.85, respectively, and was dropped immediately below 4 only in FA treated silages (Figures 1 and 2). The other treatments including control caused an only moderate decrease of the pH.

At early fermentation state, the pH of the control silage was lower than that of the silages treated with nitrite solutions in both experiments. The slowest decrease of pH was observed during the nitrite solution treatment with the highest amount of hexamine. The pH of all silages except NaHe600 had reached a pH below 4 after an ensiling period of 154 days.

Clostridia and Saccaromyces cerevisiae

Since clostridia DNA was detected only in some FA treated silages the results are presented here. The content of *C. perfringens* was 5.17 log copies g^{-1} FW in a single replicate silage in experiment 1. The content of *C. sporogenes* was 4.7 and that of *C. tyrobutyricum* 5.9 log copies g^{-1} FW in single silage replicates in experiment 2. In all other silages, no DNA were detected.

In both unwilted and wilted silages, the copy number of *S. cerevisiae* was in average higher in additive treated silages compared with untreated control silage ($P<0.001$) (Tables 3 and 4). In experiment 2 NaHe treatments increased *S. cerevisiae* compared to FA treatment ($P<0.001$). With increasing amounts of hexamine the copy number of *S. cerevisiae* curvilinearly increased in unwilted silages ($P<0.001$) and decreased in wilted silages ($P<0.05$). No correlation between *S. cerevisiae* numbers and ethanol content of the silages was observed ($R^2 = 0.04$, RMSE=2.86).

Discussion

Silage fermentation and clostridia

Additives vs untreated control

Herbage fermentation coefficient and nitrate content were below the requirements for a potentially good quality silage, proposed by Kaiser and Weiss.[37] Furthermore, the ensiling material was contaminated with clostridia. Despite this poor starting situation, major quality

differences between the treated silages and the untreated control were only in ammonia-N amounts which were much higher in the untreated silages.

Even though clostridia DNA was not detected in the untreated control, traces of butyric acid were observed. The reason might be that their DNA was metabolised after cell lysis and spore damage. The production of butyric acid in silage by other microbes than clostridia is possible but of minor importance[38]. Enterobacteria are known to reduce nitrate and deaminate amino acids.[7] Although the lactic acid content of the control silages was higher than in treated silages, the lack of fermentation inhibiting additive might have led to a slower acidification rate and the possibility for enterobacteria to proliferate in the untreated silages. That would also explain the absence of clostridia DNA and only small amounts of butyric acid. Nitrite and nitrogen oxide, products of nitrate reduction by enterobacteria, have a strong anti-microbial effect on clostridia.[1]

Sodium nitrite–hexamine based silage additive compared with formic acid

Immediately after application, FA dropped the pH of the unwilted and wilted herbage to pH 3.60 and 3.75, respectively. In untreated and NaHe silages the pH was still above 5.50 after 18 hours. Formic acid accelerated the acidification of the forage instantly after application, but with ongoing fermentation time, in the unwilted silage, the pH first started to raise and finally decreased again below 4. The raising of pH could be explained according to Spoelstra[2] by an elevation of BC during initial silage fermentation phase.

Formic acid might only extend the lag phases of microbes, but not diminish them. Thus, microbial activity at later fermentation stages probably explains higher butyric acid concentrations in the wilted FA silage compared with the NaHe silages. The observation is in line with the results of Kaiser and Weiss[37] showing that although FA dropped the pH (<4) of

cocksfoot-legume mixture and prevented lactic acid fermentation, butyric acid fermentation started 56 days after ensiling.

In the present experiment, the application rate of FA might explain why butyric acid was only found in wilted FA silages. The amount of 4 L FA t^{-1} FM equals 16.7 L t^{-1} DM in wilted forage being about 10 l less than the amount applied to the unwilted forage on DM basis. In unwilted silage, the pH decreased to less than 4 and remained there until the end of the ensiling period. The fermentation of the unwilted FA silage was very limited compared to NaHe silages as evidenced by higher residual WSC content, no lactic acid, and less acetic acid, ethanol and ammonia. The high WSC content of the FA silages, even higher than in raw material, can be partly explained by the acidic degradation of cell wall components (hemicellulose, cellulose) into soluble WSC.[39] The results suggest that the application rate of FA should be related to DM content of the forage at least if DM content is at the same level as in our experiment. Accordingly, an application rate of 4 L t^{-1} (1000 g kg^{-1}) was not able to prevent clostridial fermentation in our previous study on white lupin-wheat silages with DM contents ranging between 212 and 307 g kg^{-1}.[9] Similarly, Chamberlain and Quig[10] found also low silage quality with 4 L t^{-1} of FA (750 g kg^{-1}) with low DM of 160 g kg^{-1} in perennial rye-grass.

The nitrate content in the present trial might have been high enough to induce an elevation of BC at the initial phase of fermentation. According to Spoelstra[2] this can be explained by the consumption of protons when nitrate/nitrite is reduced to ammonia by bacteria or chemically by disproportion of nitrite to nitrate and nitrogen monoxide. The emerging ammonia and the decrease of protons will raise the silage BC and pH and allow clostridia to grow. However, in the present experiment despite low pH and almost no signs of malfermentation, elevated amounts of ammonia-N were observed in all silages except unwilted FA silage. This suggests that the characteristics of white lupine may explain extended protein degradation and ammonia

production. High ammonia values were apparently connected to high buffering capacity of the forages and high pH at the early stages of ensiling. The importance of low pH for inhibition of proteolysis by plant and microbial enzymes and thus for ammonia production is well-known[40].

Without the presence of nitrite and enterobacteria, clostridia start to form butyric acid as a fermentation end product, if the level of pH is not low enough to prevent microbial activity.[2,7] If nitrate is present, clostridia utilize nitrate as electron acceptor. The fermentation pathway to butyric acid is not necessary for recycling the reduced nicotinamide adenine dinucleotide (NADH), because nitrate is used as last electron acceptor in a respiratory chain like reaction. Thus, the fermentation product shifts from butyric acid to acetic acid, gaining more ATP from the sugar source.[41] The presence of enterobacteria and their fermentation product nitrite ends the activity of clostridia and destroys even the spores.[1]

Compared with our previous experiment where butyric acid was found in every FA silage[9], the present results were improved although the pre-ensiled forage was contaminated with clostridia. This might indicate that nitrite must be formed during fermentation or be added as additive to prevent the clostridial growth when formic acid is used or a risk for clostridia contamination is apparent. If the formation of nitrite is impaired, the risk of butyric acid formation and the surviving of clostridial spores is probable. Possibly the untreated control silages enabled the growth of enterobacteria and the moderate nitrate concentration of the herbage led to nitrite formation and only traces of butyric acid were found in the control silages. According to Spoelstra[2], enterobacteria and clostridia can use nitrate as electron sinks and reduce nitrite further to ammonia. In addition, enterobacteria also reduce nitrate to nitrite which is toxic to clostridia.[7]

Adding hexamine to sodium nitrite solution

The addition of hexamine to the nitrite solution did not improve silage quality. This is in accordance with the investigation of Knický and Spörndly.[42] They found no differences in silage quality and clostridia spores, utilising additive mixtures of sodium nitrite, sodium propionate and sodium benzoate with or without hexamine. In water hexamine dissolves under slightly acidic conditions into ammonia and formaldehyde the latter being the actual active substance.

Formaldehyde can react in many ways with amino acids and proteins and enzymes.[1] The reaction products are not degradable by enzymes and thus, this reaction should reduce protein degradation and the forming of ammonia. Considering the needs of an acidic environment and the possibility to react also with plant enzymes and proteins, the use of hexamine as silage additive might be counterproductive, especially for forages with high protein content because hexamine/formaldehyde binds to all protein compounds regardless of the origin, bacteria or plant. Therefore, the application rate of formaldehyde should be related to the protein content of the forage.[14] In our present experiment, the addition of hexamine did not reduce ammonia formation compared to NaHe0, indicating that the dose of hexamine was insufficient to prevent protein degradation even at the highest application rate.

Hellberg[12] investigated mixtures of 1500 g sodium nitrite and 2500 g hexamine per ton fresh herbage and compared the results with silages treated with 1500 g sodium nitrite per t herbage. Although the first mixture contained additional 2500 g hexamine per ton herbage, the results were not consistently better compared with solely sodium nitrite treated silages. The application rates of formaldehyde (2.5 kg t^{-1} FM) used for the experiments of Hellberg[12] were much higher than the highest application rates originated from hexamine (600 g hexamine) in our experiment. Applying formaldehyde at rates like in the experiments of Hellberg[12] and Kaiser *et al.*[14] led to low silage quality and triggered *C. tyrobutyricum* fermentation in their experiments.

A sole solution of sodium nitrite at an application rate of 900 g t^{-1} forage led to good quality silages without clostridia DNA. The effects of the addition of hexamine were not consistent. On both wilted and unwilted forages, the addition of hexamine affected fermentation resulting in linearly increasing pH-values and decreasing lactic acid concentrations. In addition, hexamine enhanced acetic acid and ethanol formation in unwilted silages which might be attributed to the better adaptation of enterobacteria to formaldehyde in the unwilted environment as suggested by Kaiser *et al.*[14] The higher DM content in our wilted silages might have enhanced the effect of hexamine to restrict enterobacteria fermentation causing decreased acetic acid and ethanol concentrations with increasing hexamine application.

Saccaromyces cerevisiae and volatile organic compounds

The copy number of *S. cerevisiae* was in average higher in additive treated silages than in untreated silages in both experiments. Inconsistent results were obtained on the effects of different additives on *S. cerevisiae*. Only in wilted silages FA was able to prevent yeast growth compared to other additives and the curvilinear effect of increasing hexamine application rate was different in the two experiments. The reason for this is not clear but might be explained by diverse conditions and/or availability of substrate for yeasts in the unwilted and wilted silages.

According to the regression analysis, elevated *S. cerevisiae* copy numbers did not generate higher ethanol concentrations. This might be related to the lack of sufficient oxygen because at least FA silages exposed high residual WSC amounts. Traces of oxygen are required for synthesizing certain membrane compounds necessary for anaerobic yeast fermentation.[43] Ethanol content in silages is the result of diverse microbial activity and their different ways to ferment nutrients. In this trial, it was impossible to determine the contribution of *S. cerevisiae* to the ethanol content of the silages.

Additive treatment reduced the sum of ethyl lactate and ethyl acetate concentrations in both experiments compared with the untreated control. In the experiment with unwilted herbage, FA treatment restricted fermentation almost completely and therefore, neither ester formation nor lactic acid fermentation was observed. The increasing addition of hexamine to the nitrate solution exposed inconsistent results on ester concentrations. In agreement with the results of Barry and Fennessy[15] and Kaiser *et al.*[14] on formaldehyde, increasing amounts of hexamine slightly restricted fermentation and formation of fermentation acids with the wilted silages. The unwilted silages exposed opposite results. These observations might be related to the fact that microbes vary in their response to formaldehyde in different conditions.[14]

According to Weiss *et al.*[44], the forming of ethyl esters correlates strongly with the amount of ethanol. This is in line with our present research in which a high correlation was detected. In our previous experiment[9], the opposite was observed. The correlation was depending on ethanol content since highest ethanol amounts (25-28 g kg^{-1} DM) did not increase the amount of esters like lower contents of ethanol.

Conclusions

White lupin - wheat bi-crop was difficult to ensile due to the high buffering capacity and high moisture content. Additives improved silage quality noticeably only by reducing silage ammonia content compared with the untreated control. Herbage nitrate content of 3.8 g kg^{-1} DM may have promoted silage quality which explains the relatively good quality of untreated silage with low concentration of butyric acid. In addition, no yeast growth was observed in control silage compared with pre-ensiling herbage. The assessment of *S. cerevisiae* quantity did not explain the different ethanol amounts in the silages.

Clostridia was detected only in some FA replicates. Based on the concentrations of silage butyric acid, formic acid treatment was less effective to prevent clostridial fermentation in wilted silages compared to NaHe treatments. This indicates that nitrite based additives would be suitable when ensiling whole crops or other forages prone to clostridial contamination.

No conclusion can be drawn on the effects of increasing hexamine application rate on clostridia activity because no differences in the amount of clostridia and butyric acid were detected between NaHe silages. Hexamine increased copy numbers of *S. cerevisiae* in unwilted and decreased in wilted silages. Overall, hexamine did not improve silage quality under the trial conditions suggesting that the addition of hexamine does not produce any additional benefits.

Acknowledgements

The authors thank the Finnish Ministry of Agriculture and Forestry and the Future Fund of the University of Helsinki for funding the experiment. We also thank the laboratory staff of the Department of Agricultural Sciences of the University of Helsinki.

508 **Table 1.** Additive treatments of silages.

509

Treatment	Abbreviation	Additive	Application rate of effective substance
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 nitrit + 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

510 cfu, colony forming unit; FM, fresh matter

511

512 **Table 2.** Chemical composition, buffering capacity, fermentation coefficient and copy
513 numbers of clostridia and *Sacharomyces cerevisiae* of the unwilted and wilted white lupin-
514 wheat bi-crop prior to ensiling (g kg^{-1} dry matter, if not otherwise stated) (n=1).

515

	Unwilted	Wilted	516
			517
Dry matter,g kg^{-1}	150	240	
Calculated DM_{min}, g kg^{-1}	304	294	518
Ash	73.9	70.4	
Crude protein	171	151	519
Soluble N, g kg^{-1} N	487	699	
NDF	437	499	520
WSC	115	111	
WSC, g kg^{-1} fresh matter	17.2	26.6	521
Starch	52.7	87.6	
DOMD	650	643	522
BC, mEq per kg DM	703	630	
BC, lactic acid	63	57	523
Nitrate	3.8	3.8	
Fermentation coefficient	29.6	39.6	524
Clostridia total, log copies g^{-1} FM	5.30	9.61	
C. perfringens, log copies g^{-1} FM	5.30	0	525
C. tyrobutyricum, log copies g^{-1} FM	0	9.61	
C. butyricum, log copies g^{-1} FM	2.30	2.60	526
C. sporogenes, log copies g^{-1} FM	0	0	
S. cerevisiae, log copies g^{-1} FM	7.43	6.81	527
			528
			529
			530

531 DM, dry matter; DM_{min} calculated minimum DM content of crop to ensure high fermentation quality of silage*;
532 NDF, neutral detergent fibre; WSC, water soluble carbohydrates; DOMD, digestible organic matter in DM; BC,
533 buffering capacity; *S.cerevisiae, Sacharomyces cerevisiae*
534 Fermentation Coefficient = DM (g kg-1)/10 + 8 x WSC (g kg-1 DM)/BC (g kg-1 DM).*
535

Table 3. The effect of additive treatment on unwilted silage fermentation quality (g kg^{-1}dry matter, if not otherwise stated) and number of *Saccharomyces cerevisiae* as log copies per g fresh matter (n = 3) (Experiment 1).

	Silage additives						Statistical significances			
	CON	FA	NaHe0	NaHe300	NaHe600	SEM	Additive vs Control	NaHe vs FA	Hex Linear	Hex Quad
Dry matter, g kg^{-1}	140	143	154	156	138	5.34	0.22	0.36	0.07	0.17
pH	3.83	3.75	3.86	3.95	4.08	0.02	0.01	<0.001	<0.001	0.43
Lactic acid	120	0.00	119	111	102	3.56	<0.001	<0.001	0.01	0.85
Acetic acid	23.9	8.77	19.3	22.1	25.3	0.83	0.001	<0.001	0.001	0.85
n-Butyric acid	0.23	0.00	0.00	0.00	0.00	0.10	non-normally distributed			
Sum C4-C6 acids	0.23	0.00	0.00	0.00	0.00	0.10	non-normally distributed			
WSC	15.7	208	11.2	13.7	18.9	2.94	<0.001	<0.001	0.10	0.73
Nitrogen	26.0	25.2	25.3	24.6	28.8	1.00	0.96	0.37	0.03	0.08
Ammonia-N, g kg^{-1}N	138	50.0	141	175	204	5.26	0.48	<0.001	<0.001	0.62
Cor Amm-N, g kg^{-1}N	138	50.0	89.3	83.3	89.0	4.10	<0.001	<0.001	0.96	0.27
Methanol	5.23	4.40	4.84	6.02	7.05	0.25	0.25	0.00	<0.001	0.83
Ethanol	14.3	1.53	6.68	9.65	18.2	0.39	<0.001	<0.001	<0.001	0.001
Ethyl lactate, mg kg^{-1}DM	351	0.00	259	271	300	12.7	<0.001	<0.001	0.04	0.60
Ethyl acetate , mg kg^{-1}DM	39.7ab	0.00[a]	0.00[a]	52.7[ab]	229[b]	15.9	non-normally distributed			
El + Ea, mg kg^{-1}DM	391	0.00	259	324	530	22.6	<0.001	<0.001	<0.001	0.03
S.cerevisiae	7.0	10.5	7.1	13.4	10.6	0.4	<0.001	0.71	<0.001	<0.001

CON, no additive; FA, formic acid 4 L (1000 g kg^{-1}) t^{-1} fresh matter (FM); NaHe, hexamethylentetramine and sodium nitrite mixture; NaHe0, sodium nitrite (900 g t^{-1} forage) without hexamine; NaHe300, sodium nitrite (900 g/t forage) with 300 g hexamine t^{-1} forage; NaHe600, sodium nitrite (900g t^{-1} forage) with 600g hexamine t^{-1} forage; Hex Linear, linear effect of hexamine addition; Hex Quad, quadratic effect of hexamine addition

S. cerevisiae, *Sacharomyces cerevisiae*; SEM, standard error of the mean; DM, dry matter; Cor Ammonia-N, deducted all nitrogen applied through additive; WSC, water-soluble carbohydrates; El + Ea, the sum of ethyl lactate and ethyl acetate

Means followed by different letters in rows are statistically different at P<0.05.

Propionic, i-butyric, i-Valeric, n-valeric and caproic acids and propanol not detected

Table 4. The effect of additive treatment on wilted silage fermentation quality (g kg^{-1}dry matter, if not otherwise stated) and number of *Saccharomyces cerevisiae* as log copies per g fresh matter (n = 3) (Experiment 2).

	Silage additives						Statistical significances			
	CON	FA	NaHe0	NaHe300	NaHe600	SEM	Additive vs Control	NaHe vs FA	Hex Linear	Hex Quad
Dry matter, g kg^{-1}	219	236	235	231	217	4.37	0.06	0.16	0.02	0.37
pH	3.92	3.90	3.94	4.03	4.18	0.02	0.007	<0.001	<0.001	0.27
Lactic acid	91.8	44.8	86.2	82.1	72.4	1.49	<0.001	<0.001	<0.001	0.16
Acetic acid	18.5	13.0	17.3	16.8	14.4	0.55	<0.001	<0.001	0.004	0.17
Propionic acid	0.07	0.23	0.03	0.00	0.13	0.09	0.76	0.13	0.47	0.49
i-Butyric acid	0.13	0.20	0.00	0.00	0.13	0.12	0.72	0.30	0.46	0.67
n-Butyric acid	0.13	1.57	0.33	0.00	0.43	0.36	0.29	0.01	0.85	0.41
Sum C4-C6 acids	0.43	1.77	0.33	0.00	0.60	0.52	0.68	0.03	0.72	0.47
WSC	21.5^{ab}	33.7^{ab}	20.1^{a}	31.4^{ab}	57.6^{b}	3.17	non-normally distributed			
Nitrogen	24.5	24.2	24.5	24.9	26.3	0.46	0.38	0.08	0.02	0.36
Ammonia-N, g kg^{-1}N	157	99.3	136	156	176	3.82	0.01	<0.001	<0.001	0.95
Cor Amm-N, g kg^{-1}N	157	99.3	101	96.3	98.3	3.69	<0.001	0.88	0.58	0.46
Methanol	3.83	3.48	3.49	4.14	4.44	0.10	0.65	0.001	<0.001	0.19
Ethanol	7.18	9.79	2.68	2.12	3.13	0.58	0.002	<0.000	0.61	0.30
Propanol	0.02	0.09	0.08	0.06	0.08	0.04	0.23	0.77	0.90	0.67
Ethyl lactate, mg kg^{-1}DM	248	178	139	97.7	96.0	8.99	<0.001	<0.001	0.007	0.10
Ethyl acetate, mg kg^{-1}DM	46.7	89.3	20.0	8.67	53.3	12.77	0.79	0.002	0.10	0.10
Ea + El, mg kg^{-1}DM	294	267	159	106	150	18.27	<0.001	<0.001	0.73	0.06
S.cerevisiae	7.2	7.1	13.4	12.0	7.1	0.5	<0.001	<0.001	<0.001	0.02

CON, no additive; FA, formic acid 4 l (1000 g kg^{-1}) t^{-1} fresh matter (FM); NaHe, hexamethylentetramine and sodium nitrite mixture; NaHe0, sodium nitrite (900 g t^{-1} forage) without hexamine; NaHe300, sodium nitrite (900 g t^{-1} forage) with 300 g hexamine t^{-1} forage; NaHe600, sodium nitrite (900g t^{-1} forage) with 600g hexamine t^{-1} forage; Hex Linear, linear effect of hexamine addition; Hex Quad, quadratic effect of hexamine addition ;*S. cerevisiae*, *Sacharomyces cerevisiae*; SEM, standard error of the mean; DM, dry matter; Cor Ammonia-N, deducted all nitrogen applied through additive;
WSC, water-soluble carbohydrates; El + Ea, the sum of ethyl lactate and ethyl acetate; i-Valeric, n-valeric and caproic acids not detected
Means followed by different letters in rows are statistically different at P<0.05.

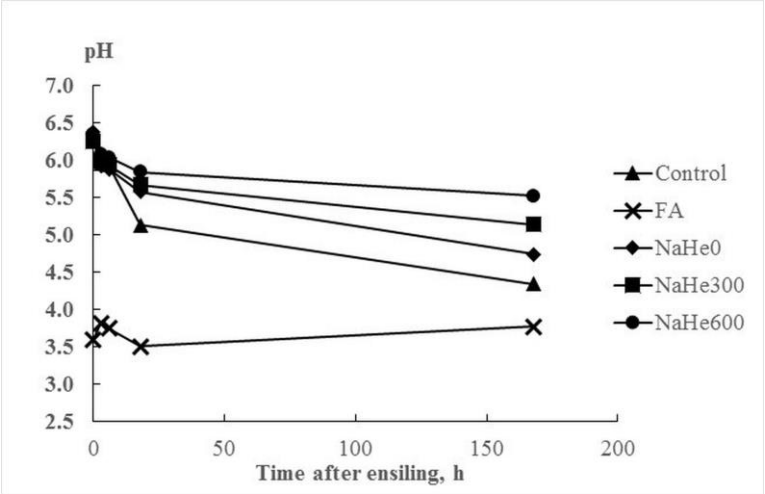


Figure 1. Unwilted silage pH after applying the additives. Control, no additive; FA, formic acid 4 L (1000 g kg^{-1}) t^{-1} fresh matter (FM); NaHe0, sodium nitrite (900 g t^{-1} forage) without hexamine; NaHe300, sodium nitrite (900 g t^{-1} forage) with 300 g hexamine t^{-1} forage; NaHe600, sodium nitrite (900 g t^{-1} forage) with 600 g hexamine t^{-1} forage

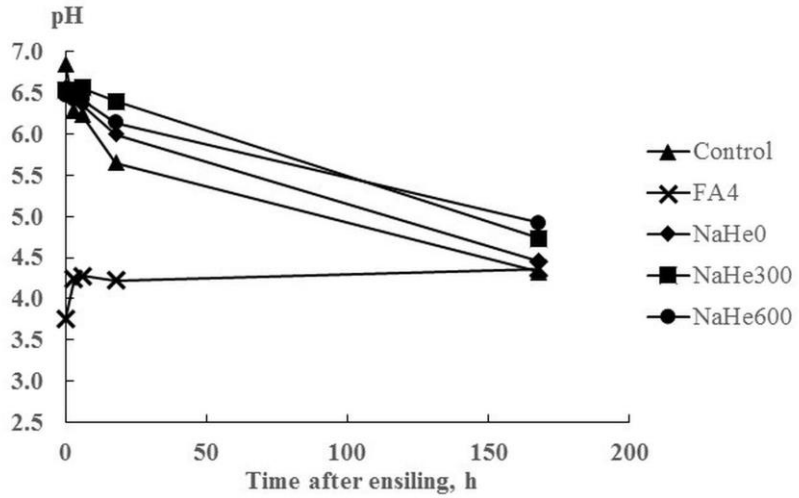
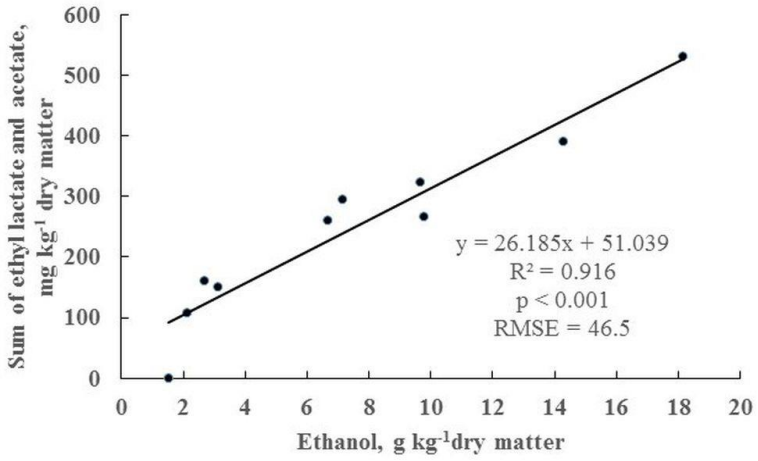


Figure 2. Wilted silage pH after applying the additives. Control, no additive; FA, formic acid 4 L (1000 g kg^{-1}) t^{-1} fresh matter (FM); NaHe0, sodium nitrite (900 g t^{-1} forage) without hexamine; NaHe300, sodium nitrite (900 g t^{-1} forage) with 300 g hexamine t^{-1} forage; NaHe600, sodium nitrite (900 g t^{-1} forage) with 600 g hexamine t^{-1} forage



569

570 **Figure 3.** Linear relationship between average the contents of ethanol and the sum of ethyl lactate

571 and ethyl acetate in silages.

572 **References**

573

574 1 Lück E and Jager M. Chemische Lebensmittelkonservierung (Chemical food preservation). 3.
575 edition. Springer-Verlag Berlin. Heidelberg. GmbH. 273 p (1995).

576

577 2 Spoelstra SF. Nitrate in silage. Grass Forage Sci. 40:1-11 (1985).

578

579 3 Wieringa GW. The influence of nitrate on silage fermentation. Proceedings of the 10th
580 International Grassland Congress, Helsinki, 1966, 537-540 (1966).

581

582 5 Lizarazo Torres C, Lampi A-M, Liu J, Sontag-Strohm T, Piironen V and Stoddard F. Nutritive
583 quality and protein production from grain legumes in a boreal climate. J. Sci Food Agric. 95: 2053-
584 2064 (2015)

585

586 4 Weiss K. Gärungsverlauf und Gärqualität von Silagen aus nitratarmem Grünfutter. Fermentation
587 process and fermentation quality of silage from green forage low in nitrate. Dissertation. Humboldt
588 Universität. 181 p (2001).

589

590 6 Azo WM, Lane GPF, Davies WP, Cannon ND. Bi-cropping white lupins (Lupinus albus L.) with
591 cereals for whole crop forage in organic farming: The effect of seed rate and harvest dates on crop
592 yield and quality. Biol. Agric. Hortic. 28: 86-100 (2012).

593

594 7 McDonald P, Henderson AR and Heron SJE. The Biochemistry of Silage. 2. ed. Chalcombe
595 Publications. Marlow, UK. 340 p (1991).

596

8 Spolders M. Anorganische Stoffe / Kontaminanten Nitrit/Nitrat. Landbauforsch Völkenrode Sonderheft 294:56-60. http://literatur.thuenen.de/digbib_extern/bitv/zi040833.pdf (2006).

9 König W, Lamminen M, Weiss K, Tuomivirta TT, Sanz Muñoz S, Fritze H et al. The effect of additives on the quality of white lupin-wheat silages assessed by fermentation pattern and q PCR quantification of clostridia. Grass Forage Sci. 72: 757-771. DOI: 10.1111/gfs.12276 (2017).

10 Chamberlain DG and Quig J. The effects of the rate of addition of formic acid and sulphuric acid on the ensilage of perennial ryegrass in laboratory silos. J. Sci. Food Agric. 87: 217-228 (1987).

11 Rammer C. Quality of grass silage infected with spores of *Clostridium tyrobutyricum*. Grass Forage Sci. 51: 88-95 (1996).

12 Hellberg A. A combination of nitrite and hexamine as an additive in the ensiling of herbage. Grass Forage Sci. 22: 289-292 (1967).

13 EFSA. European Food Safety Authority. Scientific Opinion on the safety and efficacy of hexamethylene tetramine as a silage additive for pigs, poultry, bovines, sheep, goats, rabbits and horses. EFSA Journal 13: 4014 (2015).

14 Kaiser AG, Terry RA and Dhanoa MS. Fermentation patterns in ryegrass, red clover and maize silages treated with formaldehyde additives at ensiling. J. Sci Food Agric. 32: 637-646 (1981).

15 Barry TN and Fennessy PF. The effect of formaldehyde treatment on the chemical composition and nutritive value of silage. New Zeal J. Agr Res. 15: 712-722 (1972).

16 Barry TN. Effect of treatment with formaldehyde, formic acid, and formaldehyde-acid mixtures on the chemical composition and nutritive value of silage. New Zeal J Agr Res. 18: 285-294 (1975).

17 Middelhoven WJ and van Doesburg W. Utilization of hexamethylentetramine (urotropine) by bacteria and yeast. Short communication. Anton Leeuw. Int. J. G. 91: 191-196. DOI: 10.1007/s10482-006-9103-9 (2007).

18 Dracup M and Kirby EJM. Lupine development guide. University of Western Australia Press. 97 p (1996).

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

20 AOAC. Official Methods of Analysis. 16th ed. Arlington, VA: Association of Official Analytical Chemists (1995).

21 Salo M-L and Salmi M. Determination of starch by the amyloglucosidase method. J. Scient. Agric. Soc. Finland. 40: 38-45 (1968).

22 Somogyi M A. A new reagent for the determination of sugars. J. Biol. Chem. 160: 61-63 (1945).

23 Salo, M.-L. 1965. Determination of carbohydrate fractions in animal foods and faeces. Acta Agr. Fenn. 105: 1–102.

649

650 24 McCullough H. The determination of ammonia in whole blood by direct colorimetric method.
651 Clin. Chim. Acta. 17: 297–309 (1967)

652

653 25 van Soest PJ, Robertson JB, Lewis BA. Methods of dietary fiber, neutral detergent fiber, and
654 nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597 (1991).

655

656 26 Friedel K. Die Schätzung des energetischen Futterwertes von Grobfutter mit Hilfe einer
657 Cellulasemethode. The estimation of the energetic feed value of roughage using a cellulase
658 method. Wissenschaftliche Zeitung Universität Rostock. N-Reihe 39: 78- 86 (1990).

659

660 27 Nousiainen J, Rinne M. Hellämäki M. and Huhtanen P. Prediction of the digestibility of the
661 primary growth of grass silages harvested at different stages of maturity from chemical
662 composition and pepsin-cellulase solubility. Anim. Feed. Sci. Tech. 103: 97-111 (2003).

663

664 28 Huhtanen P, Nousiainen J, Rinne M. Recent developments in forage evaluation with special
665 reference to practical applications. Agr. Food. Sci. 15: 293-323 (2006).

666

667 29 Bedwell CL, Hamar Dwayne W, Hoesterey ML, Sondermann JP, Odde KG. Comparison of
668 four methods for forage nitrate analysis. J. Vet. Diagn. Invest. 7: 527-530 (1995).

669

670 30 Weiss K and Kaiser E. Milchsäurebestimmung in Silageextrakten mit Hilfe der HPLC.
671 Determination of lactic acid in silage extracts using HPLC. Das Wirtschaftseigene Futter, 41: 69-
672 80 (1995).

673

31 Weiss K and Sommer G. Bestimmung von Estern und anderen flüchtigen organischen Substanzen (VOC) in Silageextrakten mit Hilfe der Gaschromatographie. [Determination of esters and other volatile organic substances (VOC) in silage extracts using gas chromatography]. Proc. VDLUFA- Kongress, 18. - 20. 09. 2012, Passau, Deutschland, VDLUFA-Schriftenreihe, 68: 561-569 (2012).

32 Weissbach F and Strubelt C. Correcting the dry matter content of grass silages as a substrate for biogas production. Landtechnik 63: 210-212 (2008).

33 Wilkinson J M and Davies DR. The aerobic stability of silage: Key findings and recent developments. Grass Forage Sci. 68: 1-19 (2013).

34 Hierro N, Esteve-Zarzoso, B, Mas A and Guillamon G. Monitoring of Saccharomyces and Hanseniaspora populations during alcoholic fermentation by real-time quantitative PCR. Fems Yeast Res. 7: 1340-1349 (2007).

35 Schmidt L, Weissbach F, Wernecke KD and Hein E. Erarbeitung von Parametern für die Vorhersage und Steuerung des Gärverlaufes bei der Grünfuttersilierung. Forschungsbericht. Oskar-Kellner-Institut für Tierernährung. Rostock. Deutschland (1971).

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

698 37 Kaiser E and Weiss K. Zum Gärungsverlauf bei der Silierung von nitratarmem Grünfutter 2.
699 Mitteilung: Gärungsverlauf bei Zusatz von Nitrat, Nitrit, Milchsäurebakterien und Ameisensäure.
700 Arch Tierernahr, 50: 187-200 (1997).
701
702 38 Rooke JA and Hatfield RD. Biochemistry of ensiling. In: Buxton DR, Muck RE, Harrison JH
703 (eds). Silage Science and Technology, pp. 95-139. Madison. WI: American Society of Agronomy,
704 Crop Science Society of America and Soil Science Society of America (2003).
705
706 39 Morrison, I. Changes in the cell wall components of laboratory silages and the effect of various
707 additives on these changes. J. agric. Sci., Camb., 93: 581-586. doi:10.1017/S0021859600038983
708 (1979).
709
710 40 Muck RE, Moser LE and Pitt RE. Postharvest factors affecting ensiling. In: Buxton, D.R, Muck,
711 R.E. & Harrison, J.H. (eds.). Silage Science and Technology. American Society of Agronomy;
712 Madison, USA. p. 305–360. (2003)
713
714 41 Keith SM, MacFarlane GT and Herbert R.A. Dissimilatory nitrate reduction by a strain of
715 *Clostridium butyricum* isolated fromestuarine sediments. Arch. Microbiol. 132: 63-66 (1982).
716
717 42 Knický M and Spörndly R. Sodium benzoate, potassium sorbate and sodium nitrite as silage
718 additives. J. Sci Food Agric. 89: 2659-2667 (2009).
719
720 43 O'Connor-Cox ES, Lodolo EJ and Axcell BC. Mitochondrial relevance to yeast fermentative
721 performance: a review. J. Inst. Brew. 102: 19-25 (1996).
722
723

724 44 Weiss K, Kroschewski B and Auerbach H. Effects of air exposure, temperature and additives
725 on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in
726 corn silage. J. Dairy Sci. 99: 8053-8069 (2016).

727

728

729

730