1 **Impact of hexamine addition to a nitrite based additive on fermentation quality, clostridia**

2 **and *Saccharomyces cerevisiae* in a white lupin-wheat silage**

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12

13 Abstract

14 BACKGROUND: Nitrite and hexamine are utilised as silage additives because of their adverse

15 effect on clostridia and clostridia spores. The effect of sodium nitrite and sodium

16 nitrite/hexamine mixtures on silage quality was investigated. A white lupin-wheat mixture was

17 treated with sodium nitrite (NaHe0) (900 g t^{-1} forage), or mixtures of sodium nitrite (900 g t^{-1})

18 and hexamine. The application rate of hexamine was 300 g t^{-1} (NaHe300) or 600 g t^{-1}

19 (NaHe600). Additional treatments were the untreated control (Con), and formic acid (FA)

20 applied at a rate of 4 L t^{-1}.

21 RESULTS: Additives improved silage quality noticeably only by reducing silage ammonia

22 content compared with the control. The addition of hexamine to a sodium nitrite solution did

23 not improve silage quality compared with the sole sodium nitrite solution. The increasing

24 addition of hexamine resulted in linearly rising pH values (P<0.001) and decreasing amounts

25 of lactic acid (P<0.001). Sodium nitrate based additives were more effective than formic acid

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

27 compared to the control.

28 CONCLUSION: The addition of hexamine did not improve silage quality compared with a

29 solution of sodium nitrite.

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

31

32 **Introduction**

33

34 Nitrate and nitrite are utilised as silage additives because of their adverse effect on clostridia

35 and clostridia spores.[1] In addition to additives, nitrate found naturally in forage crops affects

36 silage fermentation. Nitrate in fresh forage is reduced to several nitrogen compounds during

37 silage fermentation. Immediately after ensiling the number of nitrate reducing enterobacteria

38 increases and nitrite is accumulating.[2] Wieringa[3] ensiled grasses ranging in nitrate concentration

39 from 1 – 20 g kg^{-1} dry matter (DM). His study revealed that 4 – 8 g kg^{-1} DM nitrate in forage

40 resulted in butyric acid free silage, but silage containing both lower and higher nitrate levels

41 were prone to butyric acid fermentation. Weiss[4] found that a nitrate concentration of pre-ensiled

42 forage below 4.43 g NO_3 kg^{-1} DM exposed a higher risk for butyric acid formation in grass and

43 grass-legume silages when ensiled without silage additive.

44

45 Lupines diversify crop rotation and choice of legume plant species being an alternative for faba

46 bean and peas even in a boreal climate[5]. Especially white lupin (*Lupinus albus* L.) is a potential

47 legume to be used as whole crop silage because of its high yield[6]. However, legumes are

48 considered as difficult to ensile due to their low DM content, high buffering capacity[7] and low

49 nitrate content[8]. A former study of König *et al.*[9] revealed that a mixture of sodium nitrite and

50 hexamine was most effective to inhibit butyric acid fermentation and clostridia when different

51   mixtures of white lupine-wheat bi-crops low in nitrate content were ensiled. The administered

52   formic acid application rate (4 L t^{-1} fresh matter (FM), 1000 g kg^{-1} formic acid) was insufficient

53   to decrease pH enough for preventing the growth of clostridia and butyric acid fermentation.

54   More information is needed to control ensiling process of white lupin and other legumes

55   difficult to ensile.

56

57   The use of formic acid as additive has exposed inconsistent anti-clostridial effects on the

58   fermentation quality of silages[10]. This may be partly related to lysis of plant cells caused by

59   formic acid. Rammer[11] suggested that cell sap provides saccharolytic clostridia with nutrients

60   and enhances clostridia growth. He infected grass with spores of *Clostridium tyrobutyricum*

61   and found in silage no anti-clostridial effect of formic acid (850 g kg^{-1}) applied at a rate of 4 L

62   t^{-1} herbage.

63

64   Hellberg[12] started to investigate mixtures of nitrite and hexamine which are still used in

65   commercial products. However, there are concerns about the effects of hexamine on human

66   health.[13] From this point of view it is important to investigate the effect of hexamine on silage

67   quality. Hexamine itself has no anti-microbial effect.[1] The anti-microbial effect is based on

68   formaldehyde which is released under acidic environmental conditions from hexamine[1].

69   Formaldehyde reacts with proteins and impairs enzymes of the micro-organism. Since the effect

70   of formaldehyde is not specific to microbes, plants with high protein content may reduce the

71   efficacy of formaldehyde.[1] Utilizing formaldehyde as silage additive is well

72   investigated.[12,14,15,16] Trying to improve the effect of formaldehyde on silage fermentation

73   quality, different mixtures of formaldehyde and other additives were investigated. One of them

74   was a mixture of hexamine and sodium nitrite. Investigations of Hellberg[12] revealed that silage

75   quality of nitrite treated forages were in most cases superior to those treated with hexamine

76   alone. However, some of those trials showed a synergetic effect of hexamine and sodium nitrite.

77

78 Formaldehyde impairs lactic acid bacteria growth and induces an increasing pH[12]. On the other

79 hand, certain soil indigenous bacteria and yeasts utilize hexamine as a sole source of carbon,

80 nitrogen and energy.[17] Based on that, increasing levels of hexamine in silage might increase

81 simultaneously yeast fermentation. More information is needed on the effects of increasing

82 amounts of hexamine in mixtures with sodium nitrite on silage quality.

83

84 Two experiments were conducted to study the effects of sodium nitrite and sodium nitrite-

85 hexamine mixtures on the quality of unwilted and wilted white lupine-wheat silage compared

86 with formic acid treated and untreated silage. The main target of the study was to investigate if

87 the efficiency of sodium nitrite based additive is improved by increasing the amount of

88 hexamine in the additive. It was hypothesized that i) the use of additives prevents clostridial

89 and yeast fermentation; ii) formic acid is less effective in preventing clostridia and

90 *Saccharomyces cerevisiae* in silages than sodium nitrate or mixtures of sodium nitrite and

91 hexamine, iii) adding increasing amount of hexamine suppresses clostridia and *S. cerevisiae*

92 proliferation in silage.

93

94 **Materials and methods**

95

96 **Treatments and silage preparation**

97 White lupin (*Lupinus albus,* variety Feodora, 200 kg/ha) and spring wheat (*Triticum aestivum*

98 *L.*, variety Amaretto, 80 kg/ha) were sown as a mixture on 19 May 2014 at the Viikki Research

99 Farm of University of Helsinki, Finland (60^0N, 25^0E). The experimental field area was

100 fertilized in the previous autumn with livestock manure and in spring with a nitrogen fertilizer

101 resulting in total 50 kg N ha^{-1}.

102

103   The bi-crop was used for two separate ensiling experiments. For the experiment 1, the bi-crop

104   was harvested and ensiled unwilted on 19 August, and for the experiment 2 it was cut on 16

105   August and ensiled after 40 h wilting time. The bi-crop was harvested at a stubble height of

106   about 10 cm utilizing a disc mower (Krone EasyCut 3210 CV, Maschinenfabrik Bernard Krone

107   GmbH, Spelle, Germany). At that time, wheat was at the end of the dough stage and lupine

108   pods were filled to 75% with the green seeds. The development stage of white lupine was 4.3

109   according to the scale of Dracup and Kirby[18]. Representative samples were collected from the

110   experimental field area for botanical analyses before harvesting the bi-crop. The samples were

111   taken from six randomly chosen areas of 0.25 m^2 of size.

112

113   The forages were chopped using a laboratory chopper (Wintersteigner®, Ried im Innkreis,

114   Austria) to give a chop length of 1-4 cm. After chopping, forage was treated with the following

115   additives: 4.2 L t^{-1} formic acid (FA; Sigma Aldrich, St. Louis, USA; 950 g kg^{-1}) which equals

116   4 L pure formic acid (1000 g kg^{-1}) per ton fresh matter (FM) of forage and three mixtures of

117   sodium nitrite (Sigma Aldrich, St. Louis, USA) and hexamine (Sigma Aldrich, St. Louis, USA)

118   (NaHe). The application rates of hexamine were 0 g t^{-1} (NaHe0), 300 g t^{-1} (NaHe300) and 600

119   g t^{-1} (NaHe600) supplemented with a constant rate of 900 g t^{-1} of sodium nitrite (Table 1). The

120   control was treated with 10 mL tap water per kg FM and the additives were applied as a water

121   solution with10 mL kg^{-1} FM including additive and water. The additive was applied from a

122   spray bottle to the forage batch for each treatment and thoroughly mixed during application.

123   After additive treatment, forage samples were taken for immediate pH determination. The

124   forage was ensiled in 1.5 L glass silos (Weck®, Wher-Oflingen, Germany) with three replicates

125   per treatment. The fermentation gases were allowed to leak through the rubber seal between

126   glass silo and lid. The amount of forage filled in the silos was 1050 g (unwilted) and 900 g

127   (wilted). The density of the unwilted and the wilted compacted forage in the silos was 105 and

128 144 kg DM per m^3, respectively. Silos were stored at an ambient room temperature (20–22°C),

129 and opened 154 days after ensiling.

130

131 The same forages were ensiled also in glass silos with a volume of 120 mL to study the effect

132 of silage pH decrease at the early phase of ensiling. For each treatment, eight replicate silos

133 were used. The amount of forage filled in the silos was 90 g of unwilted forage and 80 g of

134 wilted forage, the density being 112 and 160 kg DM per m^3, respectively. The silos were sealed

135 with a rubber stopper and a screw cap. Two silos per treatment were opened 3 h, 6 h, 18 h and

136 168 h after treatment and silage pH was measured.

137

138 **Chemical analysis and aerobic stability**

139 A pre-ensiling sample of untreated bi-crop was taken for immediate DM and pH determination

140 (SevenCompactTM S220 pH, Mettler-Toledo Ltd, Leicester, Great Britain) and for later

141 analyses. Dry matter content was determined by drying the samples at 105°C for 24 h in an

142 oven (Memmert, Memmert GmbH, Schwabach, Germany). Fresh samples were frozen (-20°C)

143 for analyses of buffering capacity (BC), total and soluble nitrogen, water soluble carbohydrates

144 (WSC), nitrate and clostridia. For analyses of ash, starch, neutral detergent fibre (NDF) and *in*

145 *vitro* digestibility samples were dried at 60°C for 48 hours in a ventilated drying chamber

146 (Memmert, Memmert GmbH, Schwabach, Germany) and after that they were ground through

147 a 1-mm sieve using a laboratory mill (KT-3100, Koneteollisuus Oy, Helsinki, Finland).

148

149 After opening the 1.5 l volume silos, the content was mixed, and samples were taken for

150 immediate DM, pH and aerobic stability analyses. Samples for fermentation quality and

151 clostridia were stored at -20°C for later analyses. The silage fermentation parameters lactic acid,

152 WSC, volatile fatty acids (VFA), alcohols, acetone and ethyl esters were analysed at the

153 Humboldt Universität zu Berlin, while pH, nitrogen, ammonia-N, aerobic stability and all

154 herbage chemical analyses were made at the University of Helsinki with the methods reported

155 in detail by König et al.[9] Briefly, buffering capacity of fresh herbage was measured according

156 to Weissbach[19]. The content of N was determined by Kjeldahl method[20], and the contents of

157 herbage starch[21] and WSC[22,23] and the content of silage ammonia N[24] were analyzed by a

158 colorimetric method. Neutral detergent fibre was measured using the method of van Soest et

159 al.[25] with amylase treatment. The results were reported including residual ash. The

160 measurement of the content of digestible organic matter in DM (DOMD) was based on in vitro

161 pepsin-cellulase solubility[26] with the modifications of Nousiainen et al.[27] and the results were

162 calculated according to Huhtanen et al.[28] Forage nitrate content was measured using the

163 combined nitrate ion selective electrode (perfectION, Mettler-Toledo AG, Schwerzenbach,

164 Switzerland) and the nitrate interference suppressor solution of the manufacturer. The samples

165 were prepared for the measurement according to Bedwell et al.[29] Water-soluble carbohydrates

166 of silages were analysed by using the antron method* and lactic acid by high performance liquid

167 chromatography according to Weiss and Kaiser[30]. Volatile fatty acids (acetic, propionic,

168 isobutyric, butyric, isovaleric, valeric, and caproic acid), and alcohols (methanol, ethanol,

169 propanol) were assessed by gas chromatography (GC) according to Weiss[4] and esters (ethyl

170 lactate and ethyl acetate) by GC according to Weiss and Sommer[31].

171

172 Silage oven dried DM content was corrected for volatile substances corresponding to

173 Weissbach and Strubelt[32]: $DM_c = DM_n + (1.05 - 0.059 \times pH) \times FA + 0.08 \times LA + 0.77 \times PD$

174 $+ 0.87 \times BD + 1.00 \times AL$, where DM_c is the corrected DM, DM_n non-corrected DM, FA the

175 sum of volatile fatty acids ($C_2 - C_6$), LA lactic acid, PD 1,2-propandiol, BD 2,3-butandiol and

176 AL the sum of remaining alcohols ($C_1 - C_4$). Aerobic stability was measured over period of 12

177 days and expressed as time elapsed until the temperature rose 2°C over the ambient

178 temperature.[33]

179

180 ***Clostridium* and *Saccharomyces cerevisiae* analyses using qPCR**

181 The qPCR analyses of 4 *Clostridium* species (*C. butyricum*, *C. tyrobutyricum*, *C. sporogenes*

182 and *C. perfringens*) were conducted in the laboratory of Natural Resources Institute of Finland.

183 For each DNA extraction two to three grams of pre-ensiling herbage or silage were weighed

184 and samples were homogenized with ULTRA-TURRAX® TP-18/10 (Janke and Kunkel GmbH

185 and Co KG IKA-Werk, Staufen, Germany) in 10 mL of NEN 6877 milk-lactic-acid-glucose

186 medium. Homogenates were centrifuged using 10 000 g for 15 min at 23°C. Approximately

187 200 mg of pellet per sample was collected for DNA extraction. The DNA extraction was

188 conducted using Macherey-Nagel NucleoSpin® Soil kit (Macherey-Nagel GmbH and Co. KG**,**

189 Düren, Germany) by using SL1 lysis buffer without SX enhancer as described by the

190 manufacturer. Detailed description of the methods used in *Clostridium* analyses is given by

191 König *et al.*[9]

192

193 The DNA extraction from silage for *S. cerevisiae* analyses followed the protocol used for

194 *Clostridium* species, except following step: 30 grams of silage was homogenized in 200 mL

195 distilled water for DNA extraction.

196

197 The qPCR reactions for *S. cerevisiae* were dispensed to optical 384-wellplates (Roche

198 Diagnostics GmbH, Mannheim, Germany) using EpMotion 5070 automated pipetting system

199 (Eppendorf AG, Hamburg, Germany). Sample DNA (2.5 µL) and mixture (7.5 µL) composed

200 of $2 \times$ SYBR Green Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), primers (5

201 pmol/µL/each) and DNase/RNase free water were added into each well. Primer sequences were

202 based on Hierro *et al.*[34]

203

204 LightCycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) was used in

205 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)

232 between the treatments are expressed using different letters (a, b). The linear relationship

233 between average ethanol and the sum of ethyl lactate and ethyl acetate contents, and between

234 ethanol content and *S. cerevisiae* numbers in silages were calculated by using the REG

235 procedure of SAS (SAS 9.3, Institute Inc., Cary, NC, USA).

236

237 **Results**

238

239 **Herbage botanical and chemical composition**

240 The proportions of white lupin, wheat and weeds in the bi-crop before harvesting were on fresh

241 weight basis 0.70, 0.26 and 0.04, respectively. On DM basis, the respective values were 0.42,

242 0.51 and 0.07.

243

244 The chemical composition of the herbages prior to ensiling and the qPCR clostridia results are

245 shown in Table 2. The DM content was 150 g kg^{-1} and 240 g kg^{-1} for the unwilted and wilted

246 bi-crop, respectively. The content of WSC in DM basis was at the same level in both forages

247 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

248 calculated FC was 29.6 in the unwilted and 39.6 in the wilted bi-crop. The nitrate content was

249 the same for both wilted and unwilted bi-crop (3.8 g kg^{-1} DM).

250

251 Quantitative PCR analyses revealed the contamination of the forage with clostridia and *S.*

252 *cerevisiae* (Table 2). The unwilted forage contained 5.3 log copies g^{-1} FM of *C. perfringens*,

253 2.3 log copies g^{-1} FM *C. butyricum* and 7.43 log copies g^{-1} FM *S. cerevisiae*. The wilted forage

254 contained 9.6 log copies g^{-1} FM of *C. tyrobutyricum*, 2.6 log copies g^{-1} FM *C. butyricum* and

255 6.81 log copies g^{-1} FM *S. cerevisiae*.

256

257 **Silage aerobic stability and fermentation quality**

258 The effects of additives on fermentation quality of silages are presented in Tables 3 and 4. The

259 additive treatments are compared within experiments, not between unwilted and wilted silages

260 in experiments 1 and 2, respectively. All the 42 investigated silages were aerobically stable for

261 the whole measurement period of 12 days and therefore no results are presented.

262

263 *Additives versus untreated control*

264 The average pH of all additive treated silages was higher than in untreated control silage both

265 in the unwilted (P<0.05) and wilted (P<0.01) silages. Lactic acid and acetic acid contents were

266 lower (P<0.001) in the treated silages than in control silage in both experiments, while the

267 average WSC content of the treated silages in experiment 1 was higher compared with the

268 control silage (P<0.001). Only in experiment 2 the uncorrected ammonia-N content was lower

269 in treated silages than in control silage *(P<0.01).* However, additive treatment decreased the

270 proportion of corrected ammonia-N in in both experiments (P<0.001). The use of additives

271 resulted in lower ethanol, ethyl lactate, and the sum of ethyl lactate and ethyl acetate content in

272 all silages compared with untreated control silage (P<0.001).

273

274 *Sodium nitrite–hexamine based additives versus formic acid*

275 In experiment 1 and 2 the average pH, and contents of lactic acid and acetic acid of all NaHe

276 treated silages were higher compared with FA silages (P<0.001). Butyric acid content of NaHe

277 silages in experiment 2 was lower (P<0.05) compared with FA silage. In experiment 1 NaHe

278 treated silages contained less residual WSC than FA treated silages (P<0.001).

279

280 Unwilted NaHe silages exposed higher ammonia-N and corrected ammonia-N values compared

281 with FA treated silages (P<0.001) in experiment 1. In experiment 2 the uncorrected ammonia-

282 N content of NaHe silages was higher (P<0.001) than that of FA silages. The methanol and

283 ethanol values of NaHe silages in experiment 1 were higher than those of FA silages (P<0.01)

284 while in experiment 2 the content of methanol was higher (P<0.001) and ethanol lower in NaHe

285 than FA silages (P<0.001). The amount of ethyl acetate in experiment 1 was higher (P<0.05) in

286 NaHe0 and NaHe600 treated silage than in FA silage and lower (P<0.01) in all NaHe treated

287 silages than in FA silages in experiment 2. The content of ethyl lactate and the sum of ethyl

288 lactate and ethyl acetate in NaHe silages were higher than in FA silage in experiment 1

289 (P<0.001) and lower in experiment 2 (P<0.001).

290

291 *Addition of increasing amounts of hexamine to a sodium nitrite solution*

292 In both experiments the addition of hexamine raised linearly silage pH (P<0.001) and decreased

293 lactic acid content (P<0.05). Acetic acid values linearly increased with increasing amounts of

294 hexamine in experiment 1 (P<0.05) but decreased in experiment 2 (P<0.01). The content of

295 WSC increased between NaHe0 and NaHe600 in wilted silage (P<0.05). In both experiments

296 silage uncorrected ammonia-N proportion grew linearly with increasing hexamine application

297 rate (P<0.05).

298

299 Increasing rate of hexamine accumulated linearly the amount of methanol in silage in both

300 experiments (P<0.001), while ethanol and the sum of ethyl lactate and ethyl acetate accrued

301 curvilinearly only in experiment 1 (P<0.05). Silage ethyl lactate content increased linearly in

302 experiment 1 (P<0.05) and decreased (P<0.01) with increasing amounts of hexamine in

303 experiment 2. A strong linear relationship was found between silage ethanol and total ester

304 amounts (Figure 3).

305

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

307 In both experiments 1 and 2, the pH started to fall from an initial level of herbage pH 6.28 and

308 pH 6.85, respectively, and was dropped immediately below 4 only in FA treated silages (Figures

309 1 and 2). The other treatments including control caused an only moderate decrease of the pH.

310   At early fermentation state, the pH of the control silage was lower than that of the silages treated

311   with nitrite solutions in both experiments. The slowest decrease of pH was observed during the

312   nitrite solution treatment with the highest amount of hexamine. The pH of all silages except

313   NaHe600 had reached a pH below 4 after an ensiling period of 154 days.

314

315   *Clostridia and Saccaromyces cerevisiae*

316   Since clostridia DNA was detected only in some FA treated silages the results are presented

317   here. The content of *C. perfringens* was 5.17 log copies g^{-1} FW in a single replicate silage in

318   experiment 1. The content of *C. sporogenes* was 4.7 and that of *C. tyrobutyricum* 5.9 log copies

319   g^{-1} FW in single silage replicates in experiment 2. In all other silages, no DNA were detected.

320

321   In both unwilted and wilted silages, the copy number of *S. cerevisiae* was in average higher in

322   additive treated silages compared with untreated control silage ($P<0.001$) (Tables 3 and 4). In

323   experiment 2 NaHe treatments increased *S. cerevisiae* compared to FA treatment ($P<0.001$).

324   With increasing amounts of hexamine the copy number of *S. cerevisiae* curvilinearly increased

325   in unwilted silages ($P<0.001$) and decreased in wilted silages ($P<0.05$). No correlation between

326   *S. cerevisiae* numbers and ethanol content of the silages was observed ($R^2 = 0.04$, RMSE=2.86).

327

328   **Discussion**

329

330   **Silage fermentation and clostridia**

331   *Additives vs untreated control*

332   Herbage fermentation coefficient and nitrate content were below the requirements for a

333   potentially good quality silage, proposed by Kaiser and Weiss.[37] Furthermore, the ensiling

334   material was contaminated with clostridia. Despite this poor starting situation, major quality

335   differences between the treated silages and the untreated control were only in ammonia-N

336   amounts which were much higher in the untreated silages.

337

338   Even though clostridia DNA was not detected in the untreated control, traces of butyric acid

339   were observed. The reason might be that their DNA was metabolised after cell lysis and spore

340   damage. The production of butyric acid in silage by other microbes than clostridia is possible

341   but of minor importance[38]. Enterobacteria are known to reduce nitrate and deaminate amino

342   acids.[7] Although the lactic acid content of the control silages was higher than in treated silages,

343   the lack of fermentation inhibiting additive might have led to a slower acidification rate and the

344   possibility for enterobacteria to proliferate in the untreated silages. That would also explain the

345   absence of clostridia DNA and only small amounts of butyric acid. Nitrite and nitrogen oxide,

346   products of nitrate reduction by enterobacteria, have a strong anti-microbial effect on

347   clostridia.[1]

348

349   *Sodium nitrite–hexamine based silage additive compared with formic acid*

350   Immediately after application, FA dropped the pH of the unwilted and wilted herbage to pH

351   3.60 and 3.75, respectively. In untreated and NaHe silages the pH was still above 5.50 after 18

352   hours. Formic acid accelerated the acidification of the forage instantly after application, but

353   with ongoing fermentation time, in the unwilted silage, the pH first started to raise and finally

354   decreased again below 4. The raising of pH could be explained according to Spoelstra[2] by an

355   elevation of BC during initial silage fermentation phase.

356

357   Formic acid might only extend the lag phases of microbes, but not diminish them. Thus,

358   microbial activity at later fermentation stages probably explains higher butyric acid

359   concentrations in the wilted FA silage compared with the NaHe silages. The observation is in

360   line with the results of Kaiser and Weiss[37] showing that although FA dropped the pH (<4) of

361   cocksfoot-legume mixture and prevented lactic acid fermentation, butyric acid fermentation

362   started 56 days after ensiling.

363

364   In the present experiment, the application rate of FA might explain why butyric acid was only

365   found in wilted FA silages. The amount of 4 L FA t^{-1} FM equals 16.7 L t^{-1} DM in wilted forage

366   being about 10 l less than the amount applied to the unwilted forage on DM basis. In unwilted

367   silage, the pH decreased to less than 4 and remained there until the end of the ensiling period.

368   The fermentation of the unwilted FA silage was very limited compared to NaHe silages as

369   evidenced by higher residual WSC content, no lactic acid, and less acetic acid, ethanol and

370   ammonia. The high WSC content of the FA silages, even higher than in raw material, can be

371   partly explained by the acidic degradation of cell wall components (hemicellulose, cellulose)

372   into soluble WSC.[39] The results suggest that the application rate of FA should be related to DM

373   content of the forage at least if DM content is at the same level as in our experiment.

374   Accordingly, an application rate of 4 L t^{-1} (1000 g kg^{-1}) was not able to prevent clostridial

375   fermentation in our previous study on white lupin-wheat silages with DM contents ranging

376   between 212 and 307 g kg^{-1}.[9] Similarly, Chamberlain and Quig[10] found also low silage quality

377   with 4 L t^{-1} of FA (750 g kg^{-1}) with low DM of 160 g kg^{-1} in perennial rye-grass.

378

379   The nitrate content in the present trial might have been high enough to induce an elevation of

380   BC at the initial phase of fermentation. According to Spoelstra[2] this can be explained by the

381   consumption of protons when nitrate/nitrite is reduced to ammonia by bacteria or chemically

382   by disproportion of nitrite to nitrate and nitrogen monoxide. The emerging ammonia and the

383   decrease of protons will raise the silage BC and pH and allow clostridia to grow. However, in

384   the present experiment despite low pH and almost no signs of malfermentation, elevated

385   amounts of ammonia-N were observed in all silages except unwilted FA silage. This suggests

386   that the characteristics of white lupine may explain extended protein degradation and ammonia

387 production. High ammonia values were apparently connected to high buffering capacity of the

388 forages and high pH at the early stages of ensiling. The importance of low pH for inhibition of

389 proteolysis by plant and microbial enzymes and thus for ammonia production is well-known[40].

390

391 Without the presence of nitrite and enterobacteria, clostridia start to form butyric acid as a

392 fermentation end product, if the level of pH is not low enough to prevent microbial activity.[2,7]

393 If nitrate is present, clostridia utilize nitrate as electron acceptor. The fermentation pathway to

394 butyric acid is not necessary for recycling the reduced nicotinamide adenine dinucleotide

395 (NADH), because nitrate is used as last electron acceptor in a respiratory chain like reaction.

396 Thus, the fermentation product shifts from butyric acid to acetic acid, gaining more ATP from

397 the sugar source.[41] The presence of enterobacteria and their fermentation product nitrite ends

398 the activity of clostridia and destroys even the spores.[1]

399

400 Compared with our previous experiment where butyric acid was found in every FA silage[9], the

401 present results were improved although the pre-ensiled forage was contaminated with clostridia.

402 This might indicate that nitrite must be formed during fermentation or be added as additive to

403 prevent the clostridial growth when formic acid is used or a risk for clostridia contamination is

404 apparent. If the formation of nitrite is impaired, the risk of butyric acid formation and the

405 surviving of clostridial spores is probable. Possibly the untreated control silages enabled the

406 growth of enterobacteria and the moderate nitrate concentration of the herbage led to nitrite

407 formation and only traces of butyric acid were found in the control silages. According to

408 Spoelstra[2], enterobacteria and clostridia can use nitrate as electron sinks and reduce nitrite

409 further to ammonia. In addition, enterobacteria also reduce nitrate to nitrite which is toxic to

410 clostridia.[7]

411

412 *Adding hexamine to sodium nitrite solution*

413   The addition of hexamine to the nitrite solution did not improve silage quality. This is in

414   accordance with the investigation of Knický and Spörndly.[42] They found no differences in

415   silage quality and clostridia spores, utilising additive mixtures of sodium nitrite, sodium

416   propionate and sodium benzoate with or without hexamine. In water hexamine dissolves under

417   slightly acidic conditions into ammonia and formaldehyde the latter being the actual active

418   substance.

419

420   Formaldehyde can react in many ways with amino acids and proteins and enzymes.[1] The

421   reaction products are not degradable by enzymes and thus, this reaction should reduce protein

422   degradation and the forming of ammonia. Considering the needs of an acidic environment and

423   the possibility to react also with plant enzymes and proteins, the use of hexamine as silage

424   additive might be counterproductive, especially for forages with high protein content because

425   hexamine/formaldehyde binds to all protein compounds regardless of the origin, bacteria or

426   plant. Therefore, the application rate of formaldehyde should be related to the protein content

427   of the forage.[14] In our present experiment, the addition of hexamine did not reduce ammonia

428   formation compared to NaHe0, indicating that the dose of hexamine was insufficient to prevent

429   protein degradation even at the highest application rate.

430

431   Hellberg[12] investigated mixtures of 1500 g sodium nitrite and 2500 g hexamine per ton fresh

432   herbage and compared the results with silages treated with 1500 g sodium nitrite per t herbage.

433   Although the first mixture contained additional 2500 g hexamine per ton herbage, the results

434   were not consistently better compared with solely sodium nitrite treated silages. The application

435   rates of formaldehyde (2.5 kg t^{-1} FM) used for the experiments of Hellberg[12] were much higher

436   than the highest application rates originated from hexamine (600 g hexamine) in our

437   experiment. Applying formaldehyde at rates like in the experiments of Hellberg[12] and Kaiser *et*

438   *al.*[14] led to low silage quality and triggered *C. tyrobutyricum* fermentation in their experiments.

439

440 A sole solution of sodium nitrite at an application rate of 900 g t^{-1} forage led to good quality

441 silages without clostridia DNA. The effects of the addition of hexamine were not consistent.

442 On both wilted and unwilted forages, the addition of hexamine affected fermentation resulting

443 in linearly increasing pH-values and decreasing lactic acid concentrations. In addition,

444 hexamine enhanced acetic acid and ethanol formation in unwilted silages which might be

445 attributed to the better adaptation of enterobacteria to formaldehyde in the unwilted

446 environment as suggested by Kaiser *et al.*[14] The higher DM content in our wilted silages might

447 have enhanced the effect of hexamine to restrict enterobacteria fermentation causing decreased

448 acetic acid and ethanol concentrations with increasing hexamine application.

449

450 *Saccaromyces cerevisiae* **and volatile organic compounds**

451 The copy number of *S. cerevisiae* was in average higher in additive treated silages than in

452 untreated silages in both experiments. Inconsistent results were obtained on the effects of

453 different additives on *S. cerevisiae.* Only in wilted silages FA was able to prevent yeast growth

454 compared to other additives and the curvilinear effect of increasing hexamine application rate

455 was different in the two experiments. The reason for this is not clear but might be explained by

456 diverse conditions and/or availability of substrate for yeasts in the unwilted and wilted silages.

457

458 According to the regression analysis, elevated *S. cerevisiae* copy numbers did not generate

459 higher ethanol concentrations. This might be related to the lack of sufficient oxygen because at

460 least FA silages exposed high residual WSC amounts. Traces of oxygen are required for

461 synthesizing certain membrane compounds necessary for anaerobic yeast fermentation.[43]

462 Ethanol content in silages is the result of diverse microbial activity and their different ways to

463 ferment nutrients. In this trial, it was impossible to determine the contribution of *S. cerevisiae*

464 to the ethanol content of the silages.

465

466 Additive treatment reduced the sum of ethyl lactate and ethyl acetate concentrations in both

467 experiments compared with the untreated control. In the experiment with unwilted herbage, FA

468 treatment restricted fermentation almost completely and therefore, neither ester formation nor

469 lactic acid fermentation was observed. The increasing addition of hexamine to the nitrate

470 solution exposed inconsistent results on ester concentrations. In agreement with the results of

471 Barry and Fennessy[15] and Kaiser *et al.*[14] on formaldehyde, increasing amounts of hexamine

472 slightly restricted fermentation and formation of fermentation acids with the wilted silages. The

473 unwilted silages exposed opposite results. These observations might be related to the fact that

474 microbes vary in their response to formaldehyde in different conditions.[14]

475

476 According to Weiss *et al.*[44], the forming of ethyl esters correlates strongly with the amount of

477 ethanol. This is in line with our present research in which a high correlation was detected. In

478 our previous experiment[9], the opposite was observed. The correlation was depending on ethanol

479 content since highest ethanol amounts (25-28 g kg^{-1} DM) did not increase the amount of esters

480 like lower contents of ethanol.

481

482 **Conclusions**

483 White lupin - wheat bi-crop was difficult to ensile due to the high buffering capacity and high

484 moisture content. Additives improved silage quality noticeably only by reducing silage

485 ammonia content compared with the untreated control. Herbage nitrate content of 3.8 g kg^{-1}

486 DM may have promoted silage quality which explains the relatively good quality of untreated

487 silage with low concentration of butyric acid. In addition, no yeast growth was observed in

488 control silage compared with pre-ensiling herbage. The assessment of *S. cerevisiae* quantity did

489 not explain the different ethanol amounts in the silages.

490

491 Clostridia was detected only in some FA replicates. Based on the concentrations of silage

492 butyric acid, formic acid treatment was less effective to prevent clostridial fermentation in

493 wilted silages compared to NaHe treatments. This indicates that nitrite based additives would

494 be suitable when ensiling whole crops or other forages prone to clostridial contamination.

495

496 No conclusion can be drawn on the effects of increasing hexamine application rate on clostridia

497 activity because no differences in the amount of clostridia and butyric acid were detected

498 between NaHe silages. Hexamine increased copy numbers of *S. cerevisiae* in unwilted and

499 decreased in wilted silages. Overall, hexamine did not improve silage quality under the trial

500 conditions suggesting that the addition of hexamine does not produce any additional benefits.

501

502 **Acknowledgements**

503

507

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	
Dry matter, g kg^{-1}	150	240	517
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	520
NDF	437	499	
WSC	115	111	521
WSC, g kg^{-1} fresh matter	17.2	26.6	522
Starch	52.7	87.6	
DOMD	650	643	523
BC, mEq per kg DM	703	630	524
BC, lactic acid	63	57	
Nitrate	3.8	3.8	525
Fermentation coefficient	29.6	39.6	526
Clostridia total, log copies g^{-1} FM	5.30	9.61	
C. perfringens, log copies g^{-1} FM	5.30	0	527
C. tyrobutyricum, log copies g^{-1} FM	0	9.61	528
C. butyricum, log copies g^{-1} FM	2.30	2.60	
C. sporogenes, log copies g^{-1} FM	0	0	529
S. cerevisiae, log copies g^{-1} FM	7.43	6.81	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

536 **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*
537 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

538 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
539 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
540 600g hexamine t^{-1} forage; Hex Linear, linear effect of hexamine addition; Hex Quad, quadratic effect of hexamine addition
541 *S. cerevisiae*, *Sacharomyces cerevisiae*; SEM, standard error of the mean; DM, dry matter; Cor Ammonia-N, deducted all nitrogen applied through additive;
542 WSC, water-soluble carbohydrates; El + Ea, the sum of ethyl lactate and ethyl acetate
543 Means followed by different letters in rows are statistically different at P<0.05.
544 Propionic, i-butyric, i-Valeric, n-valeric and caproic acids and propanol not detected
545
546

547 **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
548 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

549 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
550 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)
551 with 600g hexamine t^{-1} forage; Hex Linear, linear effect of hexamine addition; Hex Quad, quadratic effect of hexamine addition ;*S. cerevisiae*, *Sacharomyces*
552 *cerevisiae*; SEM, standard error of the mean; DM, dry matter; Cor Ammonia-N, deducted all nitrogen applied through additive;
553 WSC, water-soluble carbohydrates; El + Ea, the sum of ethyl lactate and ethyl acetate; i-Valeric, n-valeric and caproic acids not detected
554 Means followed by different letters in rows are statistically different at P<0.05.

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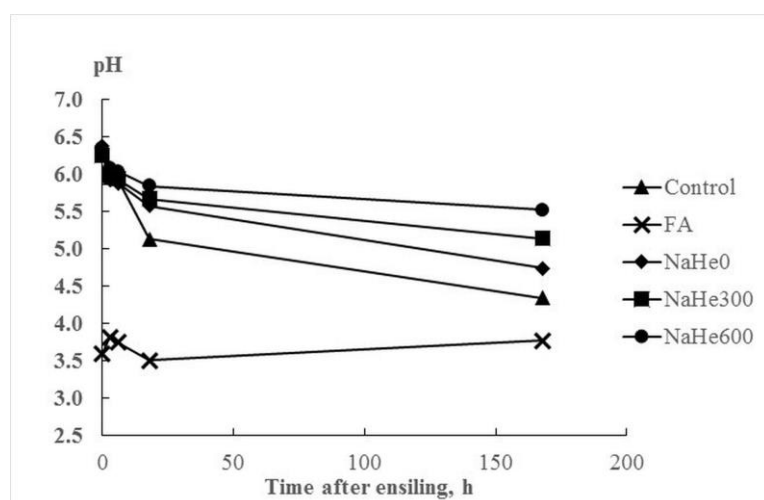
Figure 1. Unwilted silage pH after applying the additives. Control, no additive; FA, formic acid 4 L (1000 g kg^{-1}) t^{-1} fresh matter (FM); NaHe0, sodium nitrite (900 g t^{-1} forage) without hexamine; NaHe300, sodium nitrite (900 g t^{-1} forage) with 300 g hexamine t^{-1} forage; NaHe600, sodium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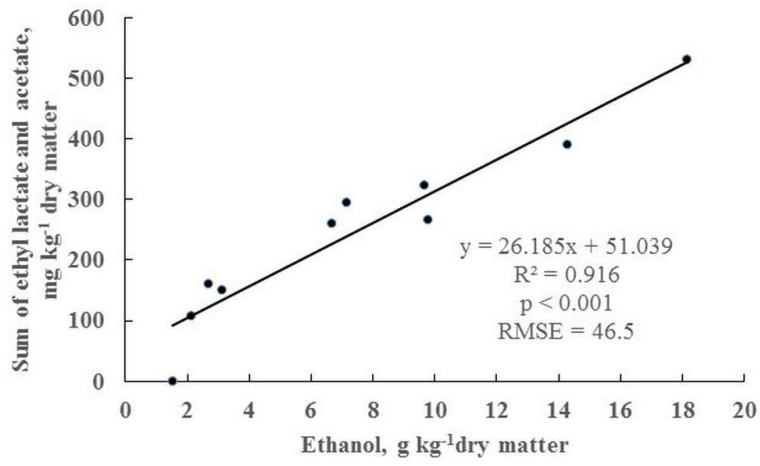
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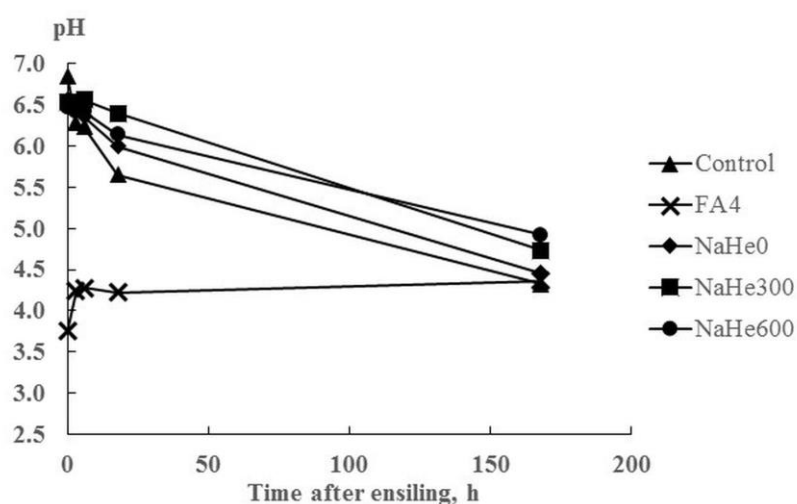
Figure 2. Wilted silage pH after applying the additives. Control, no additive; FA, formic acid 4 L (1000 g kg^{-1}) t^{-1} fresh matter (FM); NaHe0, sodium nitrite (900 g t^{-1} forage) without hexamine; NaHe300, sodium nitrite (900 g t^{-1} forage) with 300 g hexamine t^{-1} forage; NaHe600, sodium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

Figure 3. Linear relationship between average the contents of ethanol and the sum of ethyl lactate and ethyl acetate in silages.

References

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

597 8 Spolders M. Anorganische Stoffe / Kontaminanten Nitrit/Nitrat. Landbauforsch Völkenrode

598 Sonderheft 294:56-60. http://literatur.thuenen.de/digbib_extern/bitv/zi040833.pdf (2006).

599

600 9 König W, Lamminen M, Weiss K, Tuomivirta TT, Sanz Muñoz S, Fritze H et al. The effect of

601 additives on the quality of white lupin-wheat silages assessed by fermentation pattern and q PCR

602 quantification of clostridia. Grass Forage Sci. 72: 757-771. DOI: 10.1111/gfs.12276 (2017).

603

604 10 Chamberlain DG and Quig J. The effects of the rate of addition of formic acid and sulphuric

605 acid on the ensilage of perennial ryegrass in laboratory silos. J. Sci. Food Agric. 87: 217-228

606 (1987).

607

608 11 Rammer C. Quality of grass silage infected with spores of *Clostridium tyrobutyricum*. Grass

609 Forage Sci. 51: 88-95 (1996).

610

611 12 Hellberg A. A combination of nitrite and hexamine as an additive in the ensiling of herbage.

612 Grass Forage Sci. 22: 289-292 (1967).

613

614 13 EFSA. European Food Safety Authority. Scientific Opinion on the safety and efficacy of

615 hexamethylene tetramine as a silage additive for pigs, poultry, bovines, sheep, goats, rabbits and

616 horses. EFSA Journal 13: 4014 (2015).

617

618 14 Kaiser AG, Terry RA and Dhanoa MS. Fermentation patterns in ryegrass, red clover and maize

619 silages treated with formaldehyde additives at ensiling. J. Sci Food Agric. 32: 637-646 (1981).

620

621 15 Barry TN and Fennessy PF. The effect of formaldehyde treatment on the chemical composition

622 and nutritive value of silage. New Zeal J. Agr Res. 15: 712-722 (1972).

623

624 16 Barry TN. Effect of treatment with formaldehyde, formic acid, and

625 formaldehyde-acid mixtures on the chemical composition and nutritive value of silage. New Zeal

626 J Agr Res. 18: 285-294 (1975).

627

628 17 Middelhoven WJ and van Doesburg W. Utilization of hexamethylentetramine (urotropine) by

629 bacteria and yeast. Short communication. Anton Leeuw. Int. J. G. 91: 191-196. DOI:

630 10.1007/s10482-006-9103-9 (2007).

631

632 18 Dracup M and Kirby EJM. Lupine development guide. University of Western Australia Press.

633 97 p (1996).

634

635 19 Weissbach F. Bestimmung der Pufferkapazität Determination of buffering capacity of silage

636 crops. Braunschweig, Germany: Manual, Bundesforschungsanstalt für Landwirtschaft

637 Braunschweig-Völkenrode (FAL), Institut für Grünland- und Futterpflanzenforschung (1992).

638

639 20 AOAC. Official Methods of Analysis. 16th ed. Arlington, VA: Association of Official

640 Analytical Chemists (1995).

641

642 21 Salo M-L and Salmi M. Determination of starch by the amyloglucosidase method. J. Scient.

643 Agric. Soc. Finland. 40: 38-45 (1968).

644

645 22 Somogyi M A. A new reagent for the determination of sugars. J. Biol. Chem. 160: 61-63 (1945).

646

647 23 Salo, M.-L. 1965. Determination of carbohydrate fractions in animal foods and faeces. Acta

648 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

674   31 Weiss K and Sommer G. Bestimmung von Estern und anderen flüchtigen organischen

675   Substanzen (VOC) in Silageextrakten mit Hilfe der Gaschromatographie. [Determination of esters

676   and other volatile organic substances (VOC) in silage extracts using gas chromatography]. Proc.

677   VDLUFA- Kongress, 18. - 20. 09. 2012, Passau, Deutschland, VDLUFA-Schriftenreihe, 68: 561-

678   569 (2012).

679

680   32 Weissbach F and Strubelt C. Correcting the dry matter content of grass silages as a substrate

681   for biogas production. Landtechnik 63: 210-212 (2008).

682

683   33 Wilkinson J M and Davies DR. The aerobic stability of silage: Key findings and recent

684   developments. Grass Forage Sci. 68: 1-19 (2013).

685

686   34 Hierro N, Esteve-Zarzoso, B, Mas A and Guillamon G. Monitoring of Saccharomyces and

687   Hanseniaspora populations during alcoholic fermentation by real-time quantitative PCR. Fems

688   Yeast Res. 7: 1340-1349 (2007).

689

690   35 Schmidt L, Weissbach F, Wernecke KD and Hein E. Erarbeitung von Parametern für die

691   Vorhersage und Steuerung des Gärverlaufes bei der Grünfuttersilierung. Forschungsbericht.

692   Oskar-Kellner-Institut für Tierernährung. Rostock. Deutschland (1971).

693

694   36 Weissbach F. Consequences of grassland de-intensification for ensilability and feeding value

695   of herbage. Landbaufoschung Völkenrode (FAL). Contributions of Grassland and Forage research

696   to the Development of Systems of Sustainable Land Use, pp. 41-53 (1999).

697

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