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Effects of sodium diacetate and microbial inoculants on fermentation of forage rye

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Abstract

Rye (*Secale cereale* L.) is a valuable annual forage crop in Korea but there is limited information about the impact of chemical and biological additives on fermentation characteristics of the crop. This experiment was conducted to investigate fermentation dynamics of wilted forage rye treated with the following six additives; control (no additive), SDA3 (sodium diacetate applied at 3 g/kg wilted forage weight), SDA6 (6 g/kg wilted forage weight), inoculations (10^6 CFU/g wilted forage) of LP (*Lactobacillus plantarum*), LB (*L. buchneri*), or LP+LB. The ensiled rye sampled at 1, 2, 3, 5, 10, 20, 30, and 45 days indicated that the acidification occurred fast within five days of storage than the rest of the storage period. The microbial inoculants decline the pH of ensiled forage, more rapidly than the control or sodium diacetate treated, which accompanied by the decrease of water-soluble carbohydrates and increase of lactic acid. Compared with the control silage, all treatments suppressed ammonia-nitrogen formation below to 35 g/kg DM throughout the sampling period. Suppression of total microbial counting occurred in SDA6, LP, and LP + LB. The lactic acid production rates were generally higher in microbial inoculation treatments. Acetic acid concentration was lowest in the LP-treated silage and highest in the SDA- and LB-treated silages. The *in vitro* DM digestibility and total digestible nutrients were the highest in the silage treated with SDA (6 g/kg) at day 45 of ensiling. Based on lower ammonia-nitrogen concentrations and higher feed value, ensiling forage rye treated with SDA at 6 g/kg is promising through enhanced silage quality.

Keywords: Conservation; microbial inoculant; sodium diacetate; wilting; winter rye.

1. Introduction

Winter rye is an important cold- and drought-tolerant cereal crop that can remain productive in less fertile lands with a wide range of soil pH. Moreover, winter rye has several ecosystem benefits such as prevention of soil erosion and elevation of soil microorganism activities [1-4]. As a silage crop, winter rye can be harvested earlier to achieve higher quality forage (like early heading stage); early harvest also allows for another crop to be planted in a double-cropping system [5]. However, the high moisture concentration of forage rye at the vegetative stage is unsuitable for ensiling because of excessive silage effluent, soluble nutrient loss, and potential clostridial spoilage. Those factors sometimes affect the rye silage quality [6, 7]. Field wilting has been recommended to optimize the conditions for silage fermentation [8]. Wilting reduces moisture concentration in forage, minimizing protease activity, and seepage from ensiled forage [6]. Recently, Zhao et al. [9] noted that the population of lactic acid bacteria (LAB) increased when forage rye cut at the heading stage was wilted for 24 h, resulting in more desirable fermentation patterns during ensiling.

Despite the high concentration of soluble sugars in small-grain forages, the likelihood of producing high-quality silage can be increased by using biological and chemical additives, particularly when the forage dry matter (DM) concentration at ensiling is suboptimal [5, 10, 11]. Moreover, the hollow stems of winter forage rye may provide air space during ensiling and create aerobic environment in silo [12]. As a result, the use of additives is recommended to promote rapid acidification, resulting in suppression of unwanted microorganism growth at the early ensiling stages.

Sodium diacetate (SDA) is an FDA-certified preservative capable of inhibiting microbial growth and, thus improving silage fermentation quality [13, 14]. Organic acid-based additives, such as SDA, are readily ionized to acetic acid and immediately acidify forage biomass, thereby suppressing the growth and activity of unwanted organisms during the early phase of

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fermentation and minimizing the loss of nutrients during ensiling [6, 15, 16]. In their undissociated form, organic acid salts can readily permeate the cells of yeast and mold, and release hydrogen ions within the intracellular region. This increases ATP expenditure of unwanted organisms for maintenance of intracellular homeostasis, and thus disrupts their cellular metabolism [17-19].

Although the presence of epiphytic LAB on forage biomass surfaces can naturally initiate ensiling fermentation, microbial inoculants are usually recommended to support rapid acidification, which improves fermentative quality, and minimizes nutrient degradation during fermentation [20, 21]. Previous studies identified that microbial inoculants such as *Lactobacillus plantarum* (LP) and *L. buchneri* (LB), individually or in combination, enhanced the fermentative quality of cereal grain and grass silages [22-24]. However, inoculation with both homofermentative and heterofermentative LAB has provided inconsistent results when tested at variable moisture levels [25]. A meta-analysis also concluded that the improvement in fermentation quality was achieved by crop-specific application of homofermentative or heterofermentative LAB [26]. These results indicate the necessity of additional information to clarify the individual or combined effects of the LAB inoculants on the fermentation dynamics of wilted forage rye at the vegetative stage.

Information is limited comparing the usefulness of chemical- and biological-based additives for enhancing the silage fermentation quality of wilted forage rye. Therefore, this experiment was designed to determine the effects of microbial inoculants, including LP, LB, and their combination, and an organic acid preservative (SDA) on the dynamics of the anaerobic fermentation and nutrient conservation in wilted forage rye through monitoring fermentation process.

2. Materials and Methods

2.1. Forage rye production and wilting

Rye (*Secale cereale* L.) was seeded in October 2019 at the experimental field of Pyeongchang Campus, Seoul National University (37°32′46.1″ N, 128°26′17.9″ E) in Republic of Korea. The temperature and rainfall during the growing season are presented in Figure 1. Forage rye was harvested at the early heading stage (May 13, 2020) with a hand clipper. Five randomly chosen spots (1 m × 1 m) were harvested to estimate forage production. The forage rye was harvested at 6-cm stubble height and wilted on the field for 24 h, tedded in 12-h interval.

2.2. Ensiling

The wilted whole-plant rye was chopped into a theoretical cutting length of 20–30-mm using a forage cutter (Richi Machinery Co., Ltd., Henan, China) and thoroughly mixed. The forage mass was divided into six equal portions, and randomly allocated to one of the following treatments: SDA3, SDA6, LP, LB, and LP + LB. The SDA (99%; Shanghai Rhawn Chemical Technology Co., Ltd., Shanghai, China) was applied at rate of 3 (SDA3) and 6 g/kg wilted weight (SDA6). The microbial inoculants were *L. plantarum* (LP, NLRI-101), *L. Buchneri* (LB, ATCC4005), and the combination (LP + LB; 1:1 ratio) of the two lactobacilli. The application rate was 1×10^6 colonial forming unit (cfu) per g wilted mass. A control treatment (without any additives) received 10 mL distilled water per kg wilted biomass. Other five additives were sprayed uniformly on forage mass with dispensing the same volume of distilled water as the control. After manually mixing, a 400-g mass of forage was packed in a plastic vacuum bag (Food grade, 28 cm × 36 cm, Korea), sealed by a vacuum packer (FM-06; Aostar Co., Ltd., Korea). All the silage bags were stored at 20–22°C. The 0-day ensiling was sampled immediately after the preparation of all treatment silages. The stored silage treatments were

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121 opened at 1, 2, 3, 5, 10, 20, 30, or 45 days of ensiling. The sampling days were set according

122 to Santos et al. [27]. At each sampling day, the whole ensiled biomass in a bag was mixed

123 thoroughly and split into three equal amounts for later analyses. The first portion was

124 immediately frozen (–80°C) for later analysis of ammonia-nitrogen (NH_3–N), lactic acid, and

125 acetic acid. The second portion was kept fresh for immediate measurement of pH, LAB, mold,

126 and total microorganisms. The third portion was weighed fresh, dried in an oven at 65°C for

127 72 h for determination of DM and feed values.

128 *2.3. Analytical procedures*

129 Silage extract was prepared to determine the fermentation profile of rye silage [28]. The

130 acidity of the extract was determined immediately after opening the silo bags with an AB 150

131 pH meter (Fisher Scientific International, Inc., Hampton, NH, USA). Quantification of NH_3–

132 N was undertaken using a UVIDEC-610 spectrophotometer (Jasco, Tokyo, Japan) [29]. Lactic

133 acid and acetic acid were analyzed by HPLC (Detector, RI; Column, Agilent Hi-Plex H; Agilent

134 Technologies 1260 Infinity, Santa Clara, CA, USA), according to the procedure specified

135 before [30]. Lactic acid bacteria, mold, and total microorganisms were counted by streaking

136 agar plate method [31] with a detection limit of 2 $\log_{10}$ cfu/g wet weight, as described before

137 [28].

138 The dried samples were ground into 1-mm particle size using a cutting mill (Thomas

139 Scientific, Inc., New Jersey, USA) to determine nutrient value of rye silage. Neutral detergent

140 fiber (NDF) and acid detergent fiber (ADF) were analyzed sequentially [32] by adding sodium

141 sulfite and α-amylase. Analysis of total N was performed using an elemental analyzer (Euro

142 Vector EA^{3000}; EVISA, Ltd., Milan, Italy) according to the Dumas combustion method [33].

143 Acid detergent-insoluble crude protein (ADICP) was determined according to Licitra et al. [34].

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144 The anthrone method was used for analysis of water-soluble carbohydrates (WSC) [35]. *In*

145 *vitro* dry matter digestibility (IVDMD) was determined according to Goering and Van Soest

146 [36] using ANKOM Daisy[II] incubator (ANKOM Technologies, Inc., Fairport, NY, USA) [37].

147 Total digestible nutrients were calculated using the equation [88.9 – (0.79 × ADF %)] [38].

148 *2.4. Statistical analysis*

149   Data were analyzed by one-way analysis of variance (ANOVA), performed with the general

150 linear model procedure of SPSS Statistics for Windows (version 24.0; IBM Corp, Armonk, NY,

151 USA). The model used for analysis was $Y_{ij} = \mu + T_i + D_j + (T \times D)_{ij} + \varepsilon_{ij}$, where Y_{ij} = observation,

152 μ = mean, T_i = effect of treatments (silage additives), D_j = day of ensiling, $(T \times D)_{ij}$ = interaction

153 effect of treatment × day of ensiling, and ε_{ij} = error term. The experimental unit was the

154 individual ensiling bag. Before analysis, all data were tested for normality and equal variance

155 (t-test); no outliers were identified and the data were normally distributed. If treatment effect

156 was significant at $P < 0.05$, the mean differences between treatment pairs were determined by

157 Duncan's multiple range test.

3. Results and Discussion

158

159 *3.1. Pre-ensiling characteristics*

160   Heading stage is the optimum stage to forage rye because CP and other nutritive values

161 decline rapidly after this stage [7, 39]. Wilting forage rye for 24 h resulted in an increase of

162 DM concentration to 239 g/kg (Table 1). Zhao et al. [9] also noted that the DM concentration

163 was only 165 g/kg when rye was harvested at the heading stage. Therefore, 24-hour wilting

164 could increase DM concentration at a rate of 0.5 percent unit per hour to around 284 g DM /kg.

165 Kim et al. [7] also reported an increase in DM concentration from 178 to 418 g/kg when wilted

166 for 24 h. The wide variations in forage wilting from the other research indicated climate-related

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factors, namely precipitation, temperature, and wind speed, can significantly influence wilting [40]. Therefore, wilting the rye harvested around this growth stage is advantageous, considering the dry spring weather conditions in Korea.

Table 1 presents nutrient compositions and microbiological properties of the wilted rye forage treatments before ensiling. The concentrations of NDF and ADF in this experiment demonstrated substantial variation from the other study conducted with forage rye at the heading stage [41], possibly because of the differences in production management, such as seeding rate and harvesting time, as well as climate [42, 43].

Consistent with previous reports on early-cut forage rye [5], the wilted forage rye in the present experiment had a substantial WSC concentration (143 g/kg DM). This concentration was greater than the recommended minimum range (60–80 g/kg DM) for lactic acid fermentation [44]. The epiphytic LAB count in the untreated wilted rye before ensiling exceeded the recommended count of 5 $\log_{10}$ cfu/g to initiate normal silage fermentation [45].

3.2. Changes in pH, WSC, and NH$_3$–N during ensiling

The effects of additives and days of ensiling on silage pH, WSC, and NH$_3$–N concentrations of the wilted rye are presented Figure 2. Fast pH drop occurred at the early stage of ensiling (within five days), and then pH changes flattened throughout the rest of ensiling period (Fig. 2a). The pH of SDA6- and SDA3-treated silages showed steeper drops than LP, LB, and LP + LB. Previous studies on LB inoculation made pH of grass or small-grain silages higher than those of no additives [46], mainly because LB converts lactic acid to other metabolites such as acetic acid, ethanol, and 1, 2-propanediol, which cause the slight rise of the silage pH [47]. Contrarily, we could not confirm this pattern because the pH of the untreated silage was consistently higher than LB-inoculated silages throughout the monitoring period.

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190   The final pH of silage with no additives was 4.10, which is much lower than the value of

191   5.72 reported by Paradhipta et al. [42] with the untreated forage rye harvested at the dough

192   stage. This emphasizes the importance of harvest maturity for silage fermentation of forage rye.

193   Changes in DM and WSC in winter forages of different maturity can influence microbial

194   growth and silage fermentation [25].

195   The decline of WSC concentration occurred in all silage treatments, which was rapid during

196   the first 5 days of ensiling. This pattern matched to the pH declines (Fig. 2b). Silages inoculated

197   with LP, LB or LP + LB exhibited a faster rate of WSC decline during the first 5 days of

198   fermentation than other treatments. Approximately 80% of the WSC in the wilted rye was

199   utilized within 5 days of ensiling in LB, LP, and LP + LB treatments, which was significantly

200   greater than other treatments. After 45 days, the residual WSC concentration was the greatest

201   in the untreated and SDA6-treated silages. This suggests greater WSC utilization by the

202   microbial inoculants. Decline of WSC during ensiling is usually due to conversion of

203   fermentable sugars into organic acids, predominantly lactic acid by lactic fermenting bacteria

204   [6]. Alternatively, WSC could also feed undesirable enterobacteria, clostridia, acetobacter

205   bacteria, mold, and yeast, which results in a low lactic acid concentration silage. The

206   insufficient acidify could not prevent the undesirable bacteria growth [6]. In this experiment,

207   the faster decline of WSC in lactic bacteria inoculated treatments indicated higher lactic acid

208   concentration and lower silage pH.

209   In all silages, the NH_3–N concentration increased gradually as fermentation proceeded, and

210   stabilized to the final phases of ensiling (Fig. 2c). The degree of NH_3–N increase was generally

211   greater in the untreated silage than those of treatments. At day 45 of ensiling, the NH_3–N

212   concentration was lowest in SDA6-treated and LP-inoculated silages and highest in the

213   untreated silage. The protein degradation into various nitrogenous compounds during ensiling

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214 is inevitable. Especially, NH$_3$–N is low in the nutritional value [48]. The formation of NH$_3$–N

215 is accelerated by enhanced activity of microorganisms that degrade true protein fractions into

216 ammonia [6]. The NH$_3$–N concentration should be below 100 g/kg total N in silages to be

217 considered as desirable fermentation [6]. The NH$_3$–N values in this study indicated that severe

218 protein degradations did not occur during ensiling.

219 Within the early- days of ensiling, proteolysis occurred actively. The increase of NH$_3$–N

220 formation was slowest when treated with SDA6 and LP. At the early of the ensiling, a sharp

221 decline of silage pH reduced enzyme activity and microbial proteolysis [49-51]. Protease

222 begins to decrease its activity as the pH declines, with approximately 67% of the enzyme

223 activity being lost within 24 h at the pH ranging from 4 to 5 [52]. Despite the lower silage pH

224 of the LP + LB-treated silage than SDA6-treated silage, the NH$_3$–N concentration was

225 relatively higher during the ensiling process of LP + LB-treated silage, possibly because of the

226 antimicrobial properties at 6g SDA per kg wilted forage rye, which may be sufficient to

227 suppress the aerobic microorganisms activities and enzymes involved proteolysis [48]. Zhao et

228 al. [9] reported a substantially higher NH$_3$–N concentration and higher pH of wilted rye than

229 our study. The initial WSC concentration has been identified as a prerequisite for accelerating

230 the pH decline, which is also related to lower NH$_3$–N concentrations [6, 53]. In this study,

231 lactic acid concentration was higher and silage pH was lower than Zhao et al. [9], indicating

232 the higher WSC boosted lactic acid fermentation and lead to a lower silage pH, decreasing

233 protein degradation.

234 *3.2. Organic acid changes during ensiling*

235 Lactic acid formation increased gradually with ensiling, with the highest increase rates

236 within the first 5 days of fermentation (Fig. 3a). From day 0 to 3 of ensiling, lactic acid

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237 production was the highest in the LP treated silage, followed by LP + LB or LB, and the lowest

238 amount with the SDA-treated silages. Lactic acid concentration in the SDA-treated silages

239 increased slower than the inoculant treatments. Lactic acid accumulation reached its plateau

240 between 10 and 20 ensiling days, and the concentrations in LP and LP + LB were the highest

241 among the treatments at day 45 of ensiling. Lactic acid concentrations in the experimental

242 silages exceeded the typical ranges (20–40 g/kg DM), because of the high moisture content of

243 the wilted rye at ensiling (750~ 650 g/kg) promoting lactic acid formation during ensiling [54].

244 Previous research identified LP as a LAB strain whose main end-product is lactate, which is

245 mainly involved in the rapid acidifications of silage after ensiling, resulting in early suppression

246 of unwanted microorganisms and some fermentation end-products [23, 55].

247   Acetic acid presented in the highest concentration when treated with SDA at the first day of

248 ensiling (Fig. 3b). From day 2 to 5 of ensiling, acetic acid concentration increased gradually

249 followed by a slight reduction, until no significant changes to the end of ensiling period. Acetic

250 acid concentration in the LB-treated silages maintained at the typical range of 3–4% as the

251 previous report [54]. The substantially high acetic acid formation in LB-treated silage could be

252 explained with the promotion of heterofermentative metabolism in this treatment [20, 24, 56].

253 Acetic acid concentration in this study was lowest in the LP-treated silage, which is consistent

254 with the findings of Auerbach and Theobald [5], who reported higher lactic acid but lower

255 acetic acid concentrations by LP inoculation.

256   All silage treatments showed an increase in the lactic acid to acetic acid ratio during the early

257 phase of ensiling, which then decreased slightly until stabilized (Fig. 3c). This ratio was

258 generally higher for the LP- and LP + LB-treated silages, reaching its maximum value after 5

259 days of ensiling. The untreated and LB-treated silages reached their peak ratio after 10 days of

260 ensiling, while the SDA-treated silages reached their peak ratio after 20 days of fermentation.

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261 At day 45 of ensiling, the highest ratio (5.76) was seen for the LP-treated silages, while lower

262 ratios were observed in SDA6- and LB-treated silages. A lactic acid to acetic acid ratio higher

263 than 3 indicates dominant homolactic fermentation [49], and all the treatments except for SDA6

264 and LB demonstrated the ratio higher than 3.0.

265 *3.3. Microbial population changes during ensiling*

266 In all silages, LAB multiplied rapidly and generally became dominant within the first 5 days

267 of ensiling, especially in the inoculated silages, indicating that LAB was dominant in the

268 fermentation microbial community (Fig. 4a). Generally, LAB counts reached their maximum

269 on days 5–10 of ensiling, followed by a gradual decrease. For example, LAB counts in the

270 untreated and SDA3-treated silages reached their maximum after 10 days of ensiling. At day

271 45 of ensiling, LAB counts were highest in the LB-treated silages and lowest in the untreated

272 and SDA3-treated silages. The total microorganism population followed the same pattern as

273 the LAB count throughout the fermentation process (Fig. 4b). Mold count declined with

274 progression of ensiling, reaching an undetectable level in the additive-treated silages after 20

275 days of ensiling (Fig. 4c). Mold became undetectable after 45 days of ensiling in the untreated

276 silage. However, molds reached to undetectable count level faster (day 10) in the silages treated

277 with LP, LP + LB, or SDA6. In these silages, pH reduction to below 4 occurred within 5–10

278 days of ensiling (Fig. 2a). At low silage pH (~4), a greater proportion of acetate molecule exists

279 in an undissociated form, which can easily penetrate the cell membrane of yeasts and molds

280 and release hydrogen ions into the cytosol. This increases the ATP expenditures of the

281 organism needed to maintain homeostasis, thus disrupting cellular metabolism [17, 57].

282 Yuan et al. [14] reported that SDA applied at 7 g/kg fresh weight had an inhibitory effect

283 on both undesirable microorganisms and LAB development. This study also confirmed lower

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lactic acid production and lower count of LAB than other treatments when SDA was applied

at 6 g/kg wilted rye. Wen et al. [16] also observed an initial delay in lactic acid formation

compared to control silage when alfalfa (*Medicago sativa* L.) forage was treated with SDA (7

g/kg fresh weight), which was ascribed to the suppressive effect of SDA on LAB viability. The

progressive decline of LAB with longer ensiling durations is likely linked to the excessively

low silage pH and exhaustion of WSC, both of which suppress LAB growth [6, 58]. Low silage

pH suppresses the dominance of LAB, when the pH reaches 4.0 [50, 59]. These could well

explain the decline of LAB population in the later stage of ensiling in this experiment.

3.4. Nutrient value changes during ensiling

There was a gradual decline in DM concentration over the course of the ensiling process

(Fig. 5a), likely because of the loss of organic matter to water and carbon dioxide under

anaerobic conditions [6, 58].

The decrease in CP concentration during the first 3 days of ensiling in this study (Fig. 5b)

could be ascribed to the increased proteolysis caused by the activity of plant enzymes and

existing microorganisms in the initial phases of ensiling [6, 54, 60]. Ammonia is a volatile

compound and degradation of protein fractions into NH_3–N, which particularly increased in

the first 3 days of ensiling, may possibly explain the decrease of CP concentration [6]. After

day 45 of ensiling, SDA6-treated silages had the greatest CP concentration while the lowest

NH_3–N concentration, implying lower loss of the protein fractions as ammonia by the microbial

proteolysis. The lower NH_3–N concentration in SDA6-treated silage indicated that SDA had

significant effect in preserving forage proteins throughout ensiling. After 45 days, the average

ADICP concentration in all silages was 76.5 g/kg CP (Fig. 5c), which is near the threshold of

75 g/kg CP reflecting normal silage fermentation [61]. The ADICP demonstrated significant

variations among the treatments around day 20 and the variation in ADICP became narrow to a 3% range in the later phase of ensiling. A high concentration of ADICP is indicative of heat-damaged protein, which has low nutritional value for animals [62].

The effects of the additives and days of ensiling on TDN and IVDMD of the silages are presented in Table 2. The silages demonstrated inconsistency in TDN and IVDMD among the treatment across the progress of ensiling period.

At the end of ensiling, the average IVDMD was highest in the silage treated with SDA6 (840 g/kg DM), followed by SDA3, and lactic bacteria inoculation treatments. The untreated silage was lowest. The difference in IVDMD across the silages treated with microbial inoculants was not significant (mean of 817 g/kg DM). There must be decrease of non-structural carbohydrates and this reduction resulted in the relative increase of structural carbohydrate proportions, thereby lowering TDN and IVDMD as ensiling progresses [63]. Some reports proposed possible hydrolysis of fibrous carbohydrates due to the impact of accumulated acidifying agents during ensiling fermentation [6, 64], however, the inconsistency of the data do not support the theory.

4. Conclusion

Although existing conditions of the wilted rye may be sufficient to achieve lactic acid fermentation, the application of fermentation aids will carry specific benefits when the cool-season annual forage crop produces a large amount of wet biomass. As confirmed in this study, 24-hour wilting is marginally enough for moisture control. Also, the chances of unfavorable weather conditions warrant the application of silage additives to achieve enhanced forage preservation. For example, the application of sodium diacetate demonstrated its effectiveness in suppressing overall microbial activities while homolactic bacteria inoculation may help to

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boost lactic fermentation of the forage. Silage additives must be applied considering variable harvest environment and storage conditions.

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Table 1. Pre-ensiling characteristics of wilted forage rye after chemical or biological application.

Items	Treatments*						SEM
	Control	SDA3	SDA6	LP	LB	LP + LB	
Dry matter [DM, g/kg fresh weight]	239	234	234	247	248	245	1.42
Crude protein (CP, g/kg DM)	198	197	201	198	201	202	1.07
Neutral detergent fiber (NDF, g/kg DM)	525	506	502	531	528	531	2.89
Acid detergent fiber (ADF, g/kg DM)	284	279	280	287	292	294	1.41
Total digestible nutrients (%)	66.4	66.8	66.8	66.2	65.8	65.7	0.11
IVDMD (g/kg DM)	831	851	871	844	833	839	3.26
pH	6.48	6.48	6.21	6.45	6.50	6.45	0.02
Water-soluble carbohydrates (g/kg DM)	143	143	146	150	144	146	0.53
Ammonia-nitrogen (g/kg total N)	3.69	3.64	2.59	3.19	3.23	3.45	0.10
Lactic acid bacteria ($\log_{10}$ cfu/g fresh weight)	6.20	6.22	6.24	6.89	6.78	6.96	0.08
Total microorganisms ($\log_{10}$ cfu/g fresh weight)	6.49	6.55	6.49	7.20	7.15	7.42	0.09
Molds ($\log_{10}$ cfu/g fresh weight)	3.92	3.80	3.48	3.77	3.56	3.56	0.04

* Treatments were untreated silage (Control); SDA3 = sodium diacetate applied at 3 g/kg fresh weight; SDA6 = sodium diacetate applied at 6 g/kg fresh weight; LP = *L. plantarum*; LB = *L. buchneri*; LP + LB = *L. plantarum* + *L. buchneri* (1:1 ratio). Application rate of inoculants was 1×10^6 cfu/g fresh weight.
ADICP = acid detergent-insoluble crude protein; IVDMD = *in vitro* dry matter digestibility; SEM = standard error of mean.

Table 2. Effect of different additives on the dynamic change of TDN and IVDMD of wilted forage rye sampled at days of ensiling.

Items	Additives*	Days of ensiling								SEM
		1	2	3	5	10	20	30	45	
TDN (%)	Control	66.5[gB]	67.3[efBC]	67.3[efBC]	67.6[cdeC]	68.1[aC]	67.6[bcdD]	67.7[bcBC]	67.1[fC]	0.10
	SDA3	67.2[eA]	67.9[cdA]	67.7[dAB]	68.5[abA]	68.7[aB]	68.8[aA]	68.2[bcA]	67.9[cdAB]	0.11
	SDA6	67.4[dA]	67.8[cA]	68.0[bcA]	68.3[bAB]	69.5[aA]	67.8[cCD]	68.2[bA]	68.2[bA]	0.12
	LP	66.5[dB]	67.0[cC]	67.0[cCD]	68.2[aB]	68.0[aC]	67.7[bD]	66.8[cdD]	66.7[cdD]	0.13
	LB	67.4[bcA]	67.1[cdC]	66.9[dD]	67.5[bC]	68.1[aC]	68.0[aBC]	67.9[aAB]	67.9[aAB]	0.09
	LP + LB	67.2[dA]	67.5[cB]	67.6[cAB]	67.6[cC]	68.6[aB]	68.2[bB]	67.4[cdC]	67.7[cB]	0.09
IVDMD (g/kg DM)	Control	836[bB]	829[cdC]	860[aA]	827[dCD]	814[eC]	797[fgD]	835[bA]	795[gD]	4.20
	SDA3	839[bcB]	843[bB]	847[bB]	843[bAB]	825[dB]	859[aA]	828[dAB]	823[dB]	2.54
	SDA6	864[aA]	855[bA]	870[aA]	851[bA]	847[bcA]	849[bB]	836[dA]	840[cdA]	2.33
	LP	824[bcC]	837[aB]	831[abC]	841[aAB]	790[dE]	823[bcC]	819[cBC]	817[cC]	3.16
	LB	823[abC]	824[abCD]	826[aC]	824[aD]	817[bBC]	805[cD]	816[bC]	816[bC]	1.47
	LP + LB	823[bC]	819[bD]	834[aC]	835[aBC]	798[cD]	826[abC]	819[bBC]	817[bC]	2.41

* Treatments were untreated silage (Control); SDA3 = sodium diacetate applied at 3 g/kg fresh weight; SDA6 = sodium diacetate applied at 6 g/kg fresh weight; LP = *L. plantarum*; LB = *L. buchneri*; LP + LB = *L. plantarum* + *L. buchneri* (1:1 ratio). Application rate of inoculants was 1×10^6 cfu/g fresh weight.
TDN = total digestible nutrients; IVDMD = *in vitro* dry matter digestibility; SEM = standard error of mean.
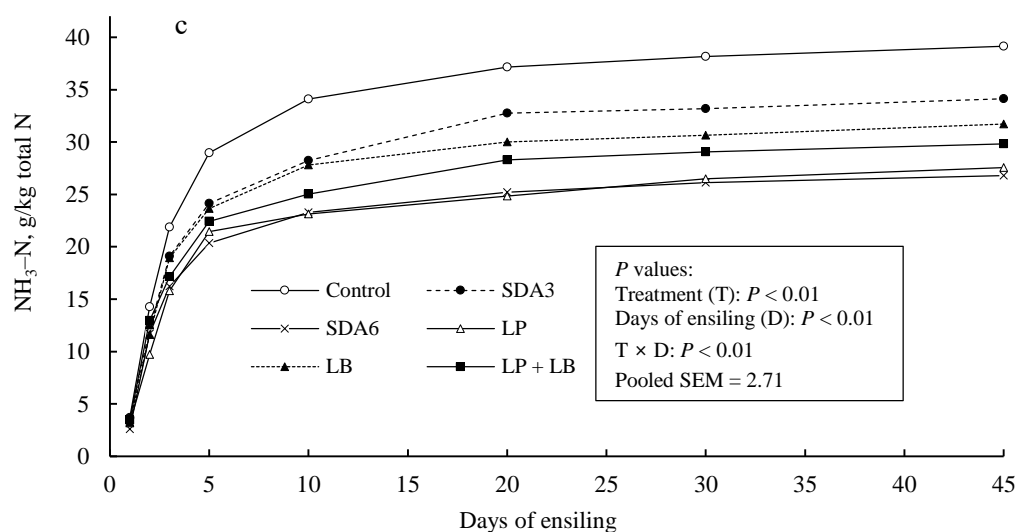[a–g] Within rows, means with dissimilar superscripts differ ($P < 0.05$).
[A–D] Within each column, means with dissimilar superscripts differ ($P < 0.05$).

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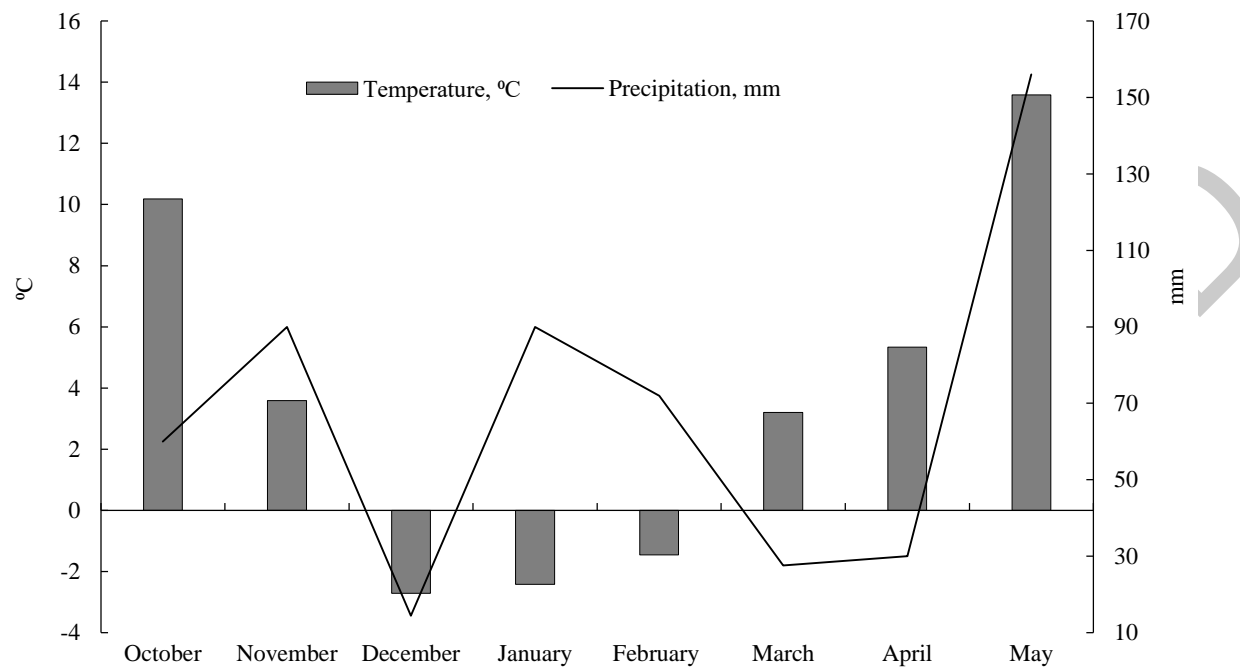
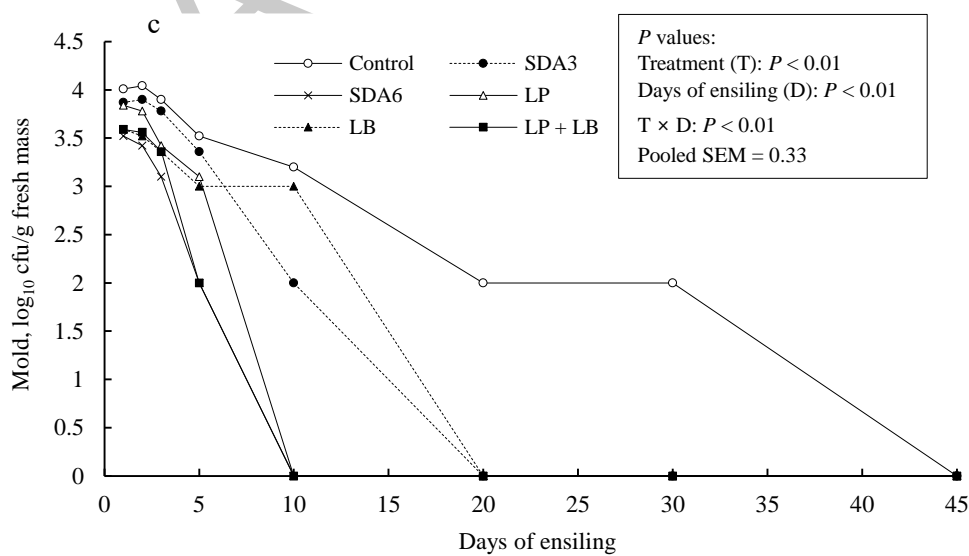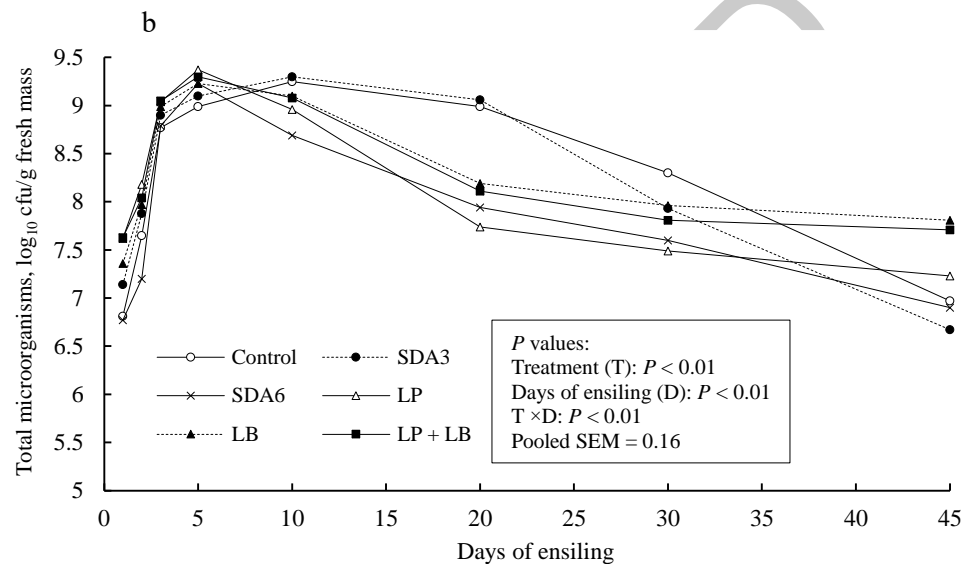**Figure 1**. Average temperature and precipitation from October 2019 to May 2020). Source: Korean Meteorological Administration.
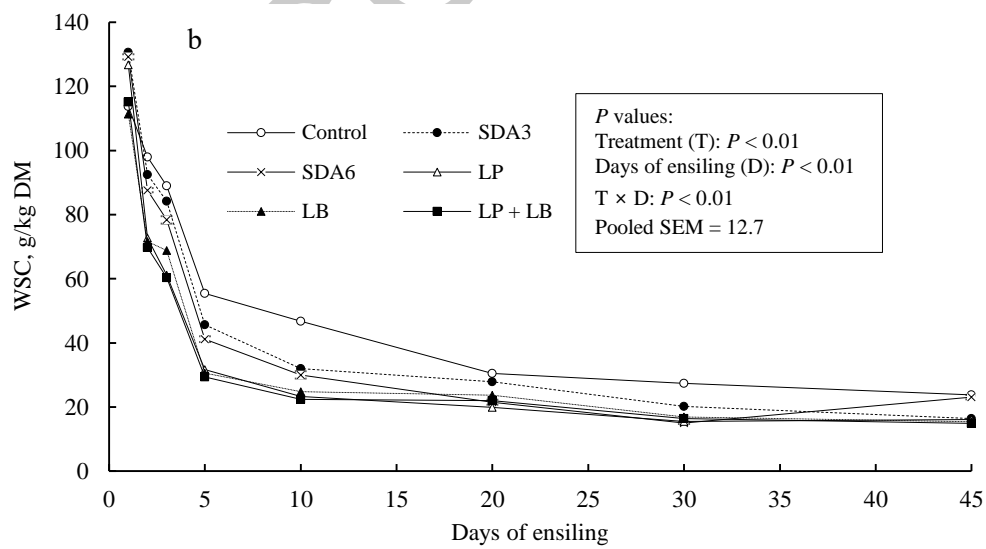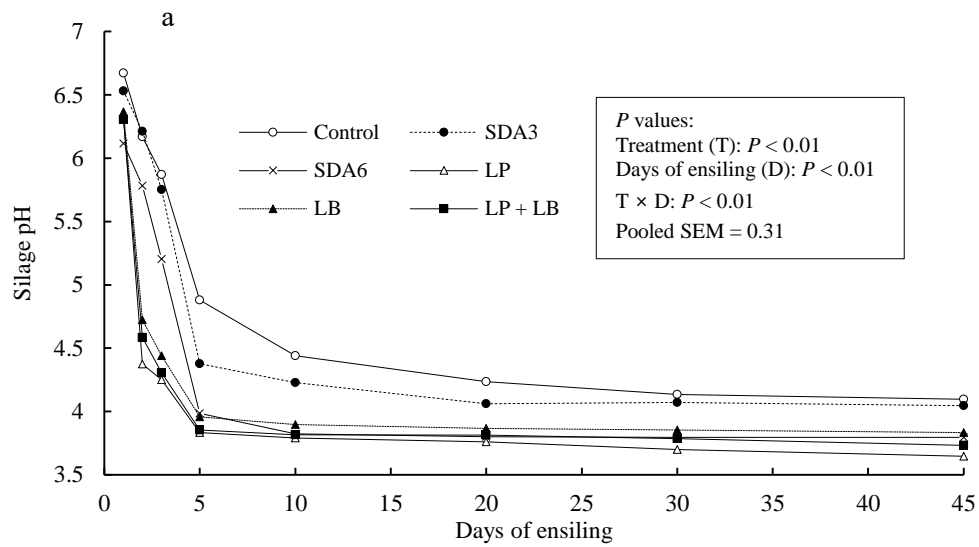
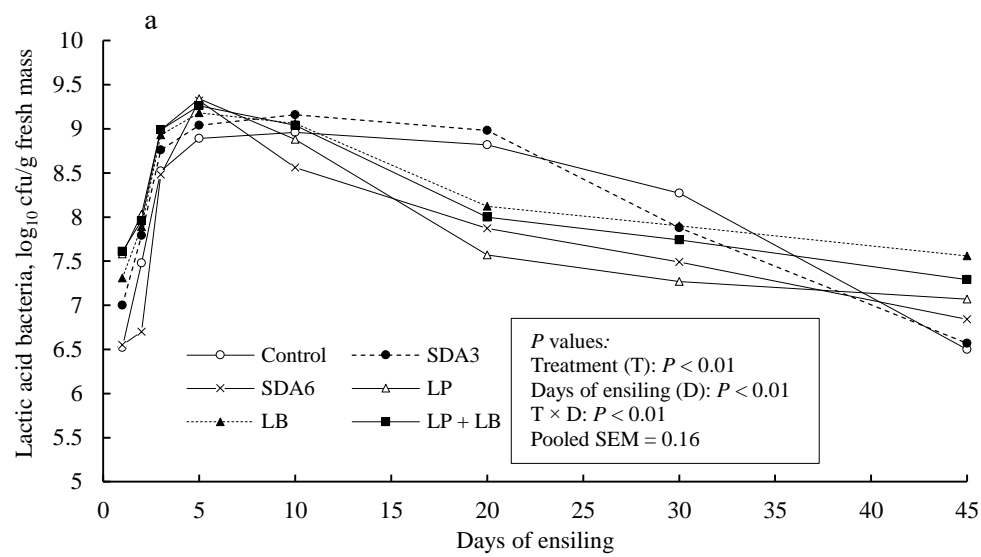**Figure 2**. Effects of additives on the dynamics of pH (a), water-soluble carbohydrates (WSC) (b), and ammonia-nitrogen (NH_3–N) concentration (c) of wilted rye silage arranged by ensiling days. Treatments were untreated silage (Control); SDA3 = sodium diacetate applied at 3 g/kg wilted weight; SDA6 = sodium diacetate applied at 6 g/kg wilted weight; LP = *L. plantarum*; LB = *L. buchneri*; LP + LB = *L. plantarum + L. buchneri* (1:1 ratio). Application rate of inoculants was 1×10^6 cfu/g wilted weight.

a

b

c
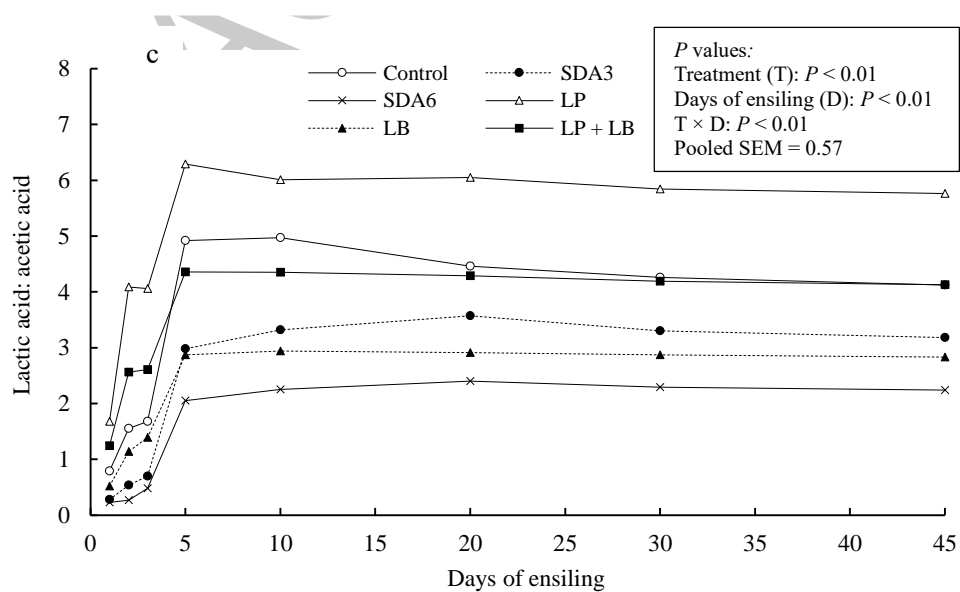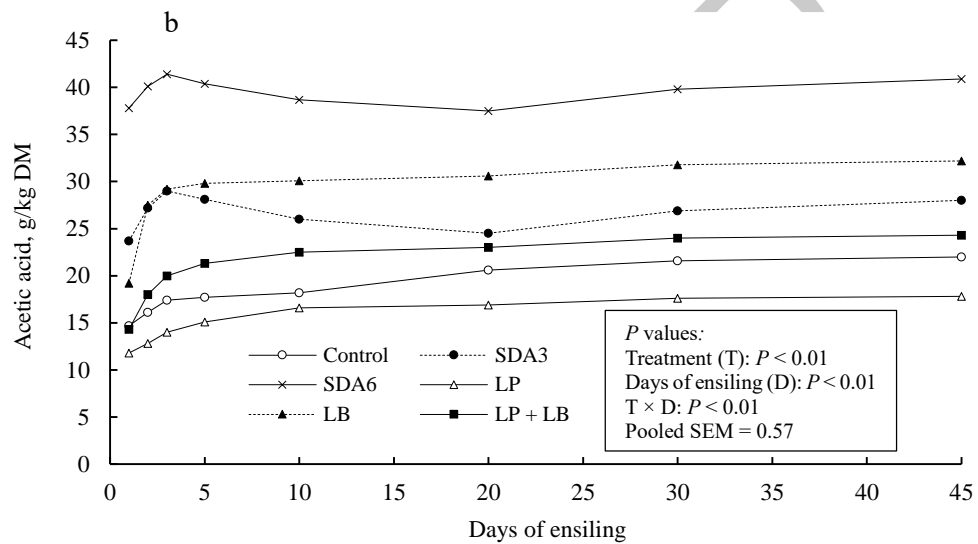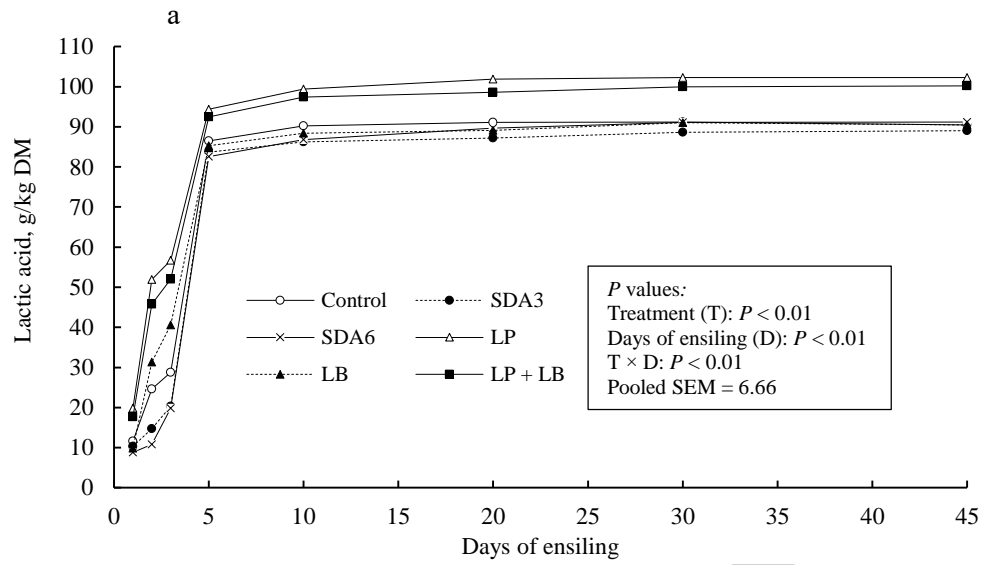
Figure 3. Effects of additives on organic acid content of wilted rye silage arranged by ensiling days; lactic acid (a), acetic acid (b), and lactic acid: acetic acid (c). Treatments were untreated silage (Control); SDA3 = sodium diacetate applied at 3 g/kg wilted weight; SDA6 = sodium diacetate applied at 6 g/kg wilted weight; LP = *L. plantarum*; LB = *L. buchneri*; LP + LB = *L. plantarum + L. buchneri* (1:1 ratio). Application rate of inoculants was 1×10^6 cfu/g wilted weight.

a

Lactic acid bacteria, $\log_{10}$ cfu/g fresh mass

P values*:*
Treatment (T): *P* < 0.01
Days of ensiling (D): *P* < 0.01
T × D: *P* < 0.01
Pooled SEM = 0.16

Control · SDA3 · SDA6 · LP · LB · LP + LB

Days of ensiling

b

Total microorganisms, $\log_{10}$ cfu/g fresh mass

P values:
Treatment (T): *P* < 0.01
Days of ensiling (D): *P* < 0.01
T ×D: *P* < 0.01
Pooled SEM = 0.16

Control · SDA3 · SDA6 · LP · LB · LP + LB

Days of ensiling

c

Mold, $\log_{10}$ cfu/g fresh mass

P values:
Treatment (T): *P* < 0.01
Days of ensiling (D): *P* < 0.01
T × D: *P* < 0.01
Pooled SEM = 0.33

Control · SDA3 · SDA6 · LP · LB · LP + LB

Days of ensiling

Figure 4. Effects of additives on the dynamics of microbial population in wilted rye silage arranged by ensiling days; lactic acid bacteria (a), total microorganisms (b), and mold (c). Treatments were untreated silage (Control); SDA3 = sodium diacetate applied at 3 g/kg wilted weight; SDA6 = sodium diacetate applied at 6 g/kg wilted weight; LP = *L. plantarum*; LB = *L. buchneri*; LP + LB = *L. plantarum* + *L. buchneri* (1:1 ratio). Application rate of inoculants was 1×10^6 cfu/g wilted weight.
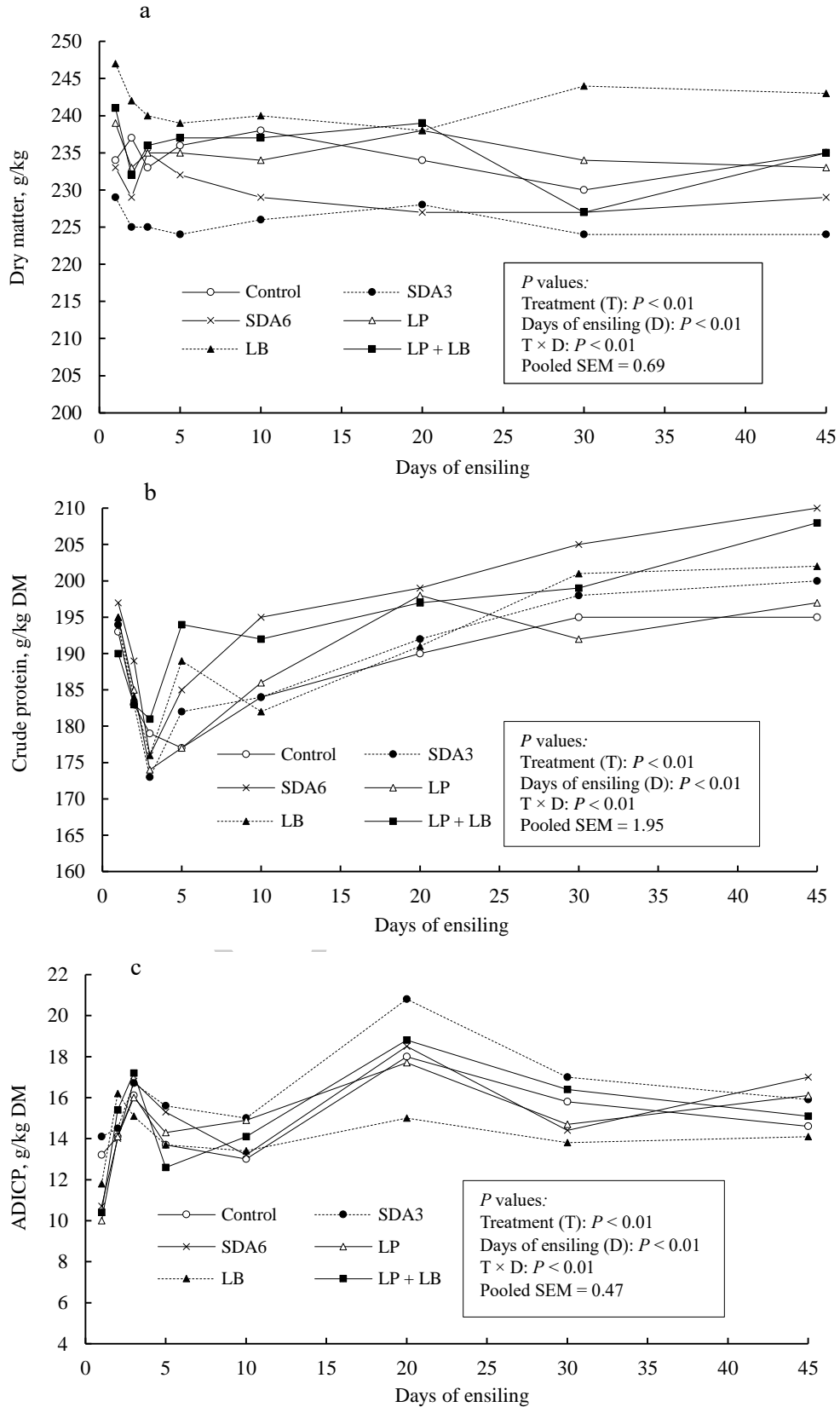
Figure 5. Effects of additives on nutritive characteristics of wilted rye silage arranged by ensiling days; dry matter (a), crude protein (b), and ADICP (c). Treatments were untreated silage (Control); SDA3 = sodium diacetate applied at 3 g/kg wilted weight; SDA6 = sodium diacetate applied at 6 g/kg wilted weight; LP = *L. plantarum*; LB = *L. buchneri*; LP + LB = *L. plantarum + L. buchneri* (1:1 ratio). Application rate of inoculants was 1×10^6 cfu/g wilted weight. SEM = standard error of mean. ADICP = acid detergent-insoluble crude protein.