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Ensiling alfalfa with hydrolyzed corn meal additive and bacterial inoculant

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SUMMARY

Authors studied the effect of hydrolyzed corn meal as additive on the fermentation of wilted alfalfa (*Medicago sativa* L.) in model ensiling experiments. Corn meal was hydrolyzed by α-amylase (BAN 480) and amyloglucosidase (Spirizyme®) and then dried. At least 89% of the corn starch was broken down to WSC (water-soluble carbohydrate). The dried product contained 587.4 g WSC/kg corn meal. Alfalfa was chopped and wilted to 323 g/kg DM before ensiling in 850 ml laboratory mini-silos and treated with an inoculant (105 cfu/g alfalfas) and 1.0, 1.5 and 2.0% hydrolyzed corn meal. Goldzyme® (a silage additive containing an inoculant and cell wall degrading enzymes) was also used, as a positive control. Treatments were ensiled in 25 replicates. Five silos for each treatment were opened after 7, 15, 30, 60 and 120 days of ensiling. The treatment with the inoculant and hydrolyzed corn, proportionally with the application rate, increased ($P < 0.05$) the lactic acid, decreased pH, and acetic acid, NH_3-nitrogen and reduced the DM loss in the silages. The use of 1% hydrolyzed corn meal was adequate to achieve a high quality, stable silage with a favourable lactic: acetic acid ratio. The quality variables of Goldzyme treated silages (pH, lactic, acetic acid and NH_3-nitrogen content) were significantly ($P < 0.05$) poorer than that of silages treated with hydrolyzed corn meal.
Keywords: alfalfa, silage, carbohydrate additive, bacterial inoculant.

INTRODUCTION

Legumes play an important role in the protein supply of ruminants and at the same time they have primary importance with other forages in the formation and maintenance of the conditions required for rumen fermentation. Alfalfa has been grown on 3.3% of the total arable land in Hungary in the last 5 years and 50–55% of the yield is used for silage. Because of its low soluble carbohydrate content and a high buffering capacity, alfalfa should be wilted and/or treated with additives before ensiling to control lactic acid fermentation.

Recently the use of additives containing lactic acid bacteria (LAB) and cell wall degrading enzymes have been gaining importance for alfalfa silage. However, reported results with these additives are inconsistent. In some cases LAB and cellulolytic enzymes resulted in improved silage quality (*Knabe et al.* 1991, *Sheperd et al.* 1995, *Rodrigues et al.* 2001). Meanwhile in others studies quality was not improved (*White et al.* 1990, *Campbell et al.* 1990, *Fredeen* and *McQueen* 1993, *Kozelov et al.* 2008). Observations with these additives on a farm scale are also controversial (*Kung et al.* 2003). These results suggest that added cellulolytic enzymes do not always provide enough fermentable sugar, (by hydrolyzing cell wall components), for the production of lactic acid, necessary to stabilize fermentation. Considering these results, there are several proposals to improve the ensiling characteristics of alfalfa by using carbohydrate supplements. When doing so, the problem aroused that traditional sugar sources (e.g. molasses) are not available on farms in several European countries because of the decrease of sugar beet production. The fermentable carbohydrate content of cereals using as additives at ensiling is very low (3–4%). Therefore high amounts of grain should be added to use them efficiently as a silage additive. For these reasons the necessary amount of fermentable carbohydrates was supplied by the enzymatic breakdown of corn starch in this experiment. However, instead of hydrolyzing "in situ" in the silo, prior to the ensiling process the hydrolysis of corn was made under a controlled environment (temperature, pH). Then the enzyme (treated, dried corn meal) was added to the alfalfa for ensilage. The objectives of the present ensiling experiment were to check the effectiveness of the hydrolyzed corn meal product as a carbohydrate source and to collect data on the necessary amount of hydrolyzed corn meal required to stabilize fermentation when ensiling wilted alfalfa.

Materials and methods

Ensiling experiments

Fresh cut alfalfa *(Medicago sativa)* was chopped and wilted to 323 g/kg DM content then 400–420 g of this chopped alfalfa was measured into 850 ml laboratory silos for the ensiling process. Water-soluble carbohydrate content of alfalfa was 29.5 g/kg which means that it was a moderately difficult to ensile forage, as defined in the European Union Regulation 429/2008 (*EU* 2008). Treatments were ensiled in 25 replicates; five silos for each treatment were opened after 7, 15, 30, 60 and 120 days of ensiling. Silage samples were processed and analyzed for pH, lactic acid, VFA, alcohol, NH_3-nitrogen and water-soluble carbohydrate (WSC) content. On the last day of ensiling (day 120) dry matter loss was determined in the silos.

The starch of the corn meal was hydrolyzed in two consecutive phases with α-amylase (BAN 480) and amyloglucosidase (Spirizyme) – both Novo products (NOVO Nordisk A/S, Denmark). This method was justified by the different conditions required by the two enzymes in terms of temperature, pH, and the duration of the hydrolysis (α-amylase: pH 5.6–6.0, at 80 °C for 20 minutes, amyloglucosidase: pH 4.5, 60 °C for 20 hours). The

amount of enzymes calculated as 1 g/kg starch. The dry matter content of the hydrolyzed corn influences the efficiency of the hydrolysis, therefore the dry matter content of the corn was set to 30%, so starch could be hydrolyzed to water-soluble carbohydrates with an approximate efficiency of 89%. The following treatments were used: control (C); inoculation (I); inoculation + 1.0% hydrolyzed corn meal (H1); inoculation + 1.5% hydrolyzed corn meal (H1.5); inoculation + 2.0% hydrolyzed corn meal (H2).
The inoculation rate was 105 cfu/g with an inoculant consisted of *Lactobacillus plantarum, Enterococcus faecium, Lactobacilllus buchneri* and *Propionibacterium freudenreichii subsp. Shermanii*. As a positive control (G), a treatment with the additive Goldzyme (Medipharm, Slovakia s.r.o.) was also included, containing an inoculant *(Enterococcus faecium, Lactobacillus plantarum, Lactobacillus casei* and *Pediococcus pentosaceus,* 1.5 x 105 cfu/g) as well as cellulase and hemicellulase enzymes (activity 0.17 IU/g).

Chemical analysis

Chemical analysis of the alfalfa was determined according to *AOAC* (1990).
Silage water extracts were analysed for lactic acid, volatile fatty acids and alcohol by HPLC (Biotronic 2000, Wissenschaftliche Geräte GmbH, Germany, Maintal 1.) Column: Bio-Rad Aminex® HPX-874, 300 mm x 7.8 mm. Separation temperature 45 °C. Eluent: 0.005M H_2SO_4, Flow: 0.85 ml/min, press 77 kg/cm^2.
Ammonia-nitrogen was determined in the water extract by NH_3 sensitive electrode (OP-2642/2, Radelkis, Hungary). Water-soluble carbohydrates were determined by the method described by *McDonald* and *Henderson* (1964).

Statistical analysis

Data were analyzed by analysis of variance by one-way ANOVA with in the SPSS 12.0 for Windows (SPSS Inc.). The homogeneity of variances between groups was previously verified by the Levene's test. The LSD post hoc test was used when variances were equal between groups and/or Dunnett's T3 was used if variances were not equal. Differences were declared significant at $P < 0.05$.

Results and discussion

The chemical composition of the silages is summarised in *Table 1.* Results show that inoculation alone (I) had a favourable effect on the quality variables of the silage only in the first 30 days of fermentation. There was a significantly lower pH, higher lactic acid, lower NH_3-nitrogen and alcohol content as compared to the negative control. However, during later period of ensiling these positive effects disappeared. These results are in agreement with those of *Kung et al.* (1987), *Satter et al.* (1987) and *Zhang et al.* (2009), who also reported an increase of lactic acid and a decrease of NH_3-nitrogen in the silage caused by inoculation with LAB. The addition of hydrolyzed corn meal to the wilted alfalfa

significantly resulted in a dose dependant increase of the lactic acid content compared to the C and to I groups. The difference in the lactid acid was significant as early as on the 7 of the ensiling day and it remained so throughout the whole duration of fermentation (120. day). The H1, H1.5 and H2 treatments decreased the acetic acid content of the silages, improving the lactic: acetic acid ratio in the treatment groups (C: 56.2-43.8%; I: 56.8-43.2%; H1:71.1-28.9%; H1.5: 74.2-25.8%; H2:80.1-19.9% and G: 59.4-40.6%, respectively).

Table 1. Measured chemical composition of alfalfa

Nutrient content	g/kg alfalfa
Dry matter	323.00
Crude protein	61.70
Crude fat	5.48
Crude fibre	96.17
Crude ash	33.03
N-free extract	126.62
NDF	133.44
ADF	102.83
ADL	22.33
WSC	29.50

The combination of the inoculant and hydrolyzed corn meal decreased the NH3–nitrogen content compared to the C and I silages. However, the combination did not affect significantly the alcohol content. This compound was increased by increasing dosage of hydrolyzed corn meal (1.5 and 2.0%) in the second half of fermentation. Results of the experiments indicated that the addition of 1% hydrolyzed corn meal is already sufficient to attain a good quality, stable silage with a favourable lactic: acetic acid ratio, when the ensiled alfalfa has a DM of 320 g/kg.

Changes in the WSC content of ensiled alfalfa and that of hydrolyzed corn meal treated silages during fermentation are shown in *Table 2*. Data suggest that the majority of WSC were used up during the first 7 days of fermentation. An increase of carbohydrate concentration moderated the utilisation of fermentable carbohydrates, resulting more residual sugar in the silages. The increased addition of hydrolyzed corn meal also moderated the use of sugar during fermentation, although the difference was not high showing 1.5–3.0% as an average.

Data of DM loss resulted by the treatments are summarized in *Figure 1.* Fermentation losses measured are in agreement with the analytical results *(Table 3.).* The combination of carbohydrate supplement and inoculation, not only improved the quality of the silages, but also reduced the losses. Treatment I resulted decrease of fermentation losses, which were further moderated by the combination of inoculant and hydrolyzed corn meal. The H1, H1.5 and H2 supplements reduced the DM loss by 4.8–5.6%, depending on the amount of hydrolyzed corn starch was used. That means a considerable relative reduction of 39.6–46.3% in DM losses.

Table 2. Impact of hydrolyzed corn additive on the fermentation of alfalfa

Treatments	days	pH	lactic acid	acetic acid	propionic acid	i-butyric acid	alcohol	NH_3-N
		% of dry matter						% of total nitrogen
C	7	5.01±0.02^{a}	3.90±0.09^{a}	1.67±0.03^{a}	traces	–	0.34±0.03^{a}	6.12±0.50^{a}
	15	5.02±0.01^{a}	4.18±0.09^{a}	2.32±0.12^{a}	0.03±0.01	0.12±0.03	0.43±0.06^{a}	8.44±0.50^{a}
	30	5.03±0.02^{a}	4.40±0.06^{a}	2.85±0.06^{a}	0.06±0.03	0.12±0.03	0.40±0.03^{a}	8.75±1.19^{a}
	60	4.92±0.01^{a}	4.83±0.22^{a}	3.34±0.09^{a}	0.09±0.03	0.12±0.03	0.40±0.03^{a}	9.19±0.31^{a}
	120	4.92±0.02^{ab}	5.08±0.15^{a}	3.96±0.15^{a}	0.12±0.03	0.09±0.00	0.65±0.06^{ac}	14.81±0.62^{a}
I	7	4.97±0.02^{b}	4.74±0.12^{b}	1.70±0.06^{a}	traces	traces	0.28±0.00^{b}	5.75±0.44^{a}
	15	4.99±0.03^{a}	5.26±0.15^{b}	2.07±0.06^{a}	traces	0.12±0.03	0.31±0.00^{bc}	6.69±0.56^{b}
	30	5.05±0.02^{a}	4.95±0.19^{b}	2.79±0.12^{a}	traces	0.12±0.03	0.34±0.03^{b}	9.31±0.75^{b}
	60	4.96±0.02^{a}	5.05±0.09^{a}	3.31±0.06^{ab}	0.09±0.03	0.12±0.00	0.37±0.03^{a}	9.19±0.75^{ab}
	120	4.95±0.02^{b}	5.20±0.43^{a}	3.96±0.09^{a}	0.12±0.03	0.12±0.03	0.46±0.09^{ab}	14.37±0.87^{a}
H1	7	4.56±0.02^{c}	6.19±0.22^{c}	1.21±0.06^{b}	–	0.09±0.00	0.31±0.03^{ab}	4.44±0.44^{b}
	15	4.52±0.04^{b}	6.50±0.15^{c}	1.46±0.06^{b}	traces	0.09±0.03	0.34±0.03^{c}	5.69±0.44^{c}
	30	4.57±0.04^{b}	6.63±0.19^{c}	1.80±0.09^{b}	0.03±0.03	0.09±0.03	0.37±0.00^{ab}	6.31±0.31^{c}
	60	4.62±0.06^{b}	6.69±0.09^{b}	2.63±0.12^{c}	0.03±0.03	0.09±0.03	0.37±0.03^{a}	6.87±0.44^{c}
	120	4.57±0.03^{c}	7.00±0.43^{b}	2.85±0.25^{b}	0.03±0.03	0.12±0.03	0.43±0.03^{b}	8.56±1.00^{b}
H1.5	7	4.41±0.02^{d}	6.59±0.12^{c}	1.08±0.03^{c}	traces	–	0.37±0.03^{a}	3.62±0.44^{c}
	15	4.39±0.03^{c}	6.87±0.22^{cd}	1.24±0.00^{c}	traces	–	0.37±0.03^{abc}	4.37±0.25^{d}
	30	4.38±0.01^{c}	7.37±0.12^{d}	1.46±0.06^{c}	traces	0.09±0.03	0.40±0.03^{ab}	4.62±0.19^{d}
	60	4.37±0.03^{c}	7.49±0.09^{c}	1.83±0.06^{d}	traces	0.09±0.03	0.65±0.03^{b}	4.56±0.44^{d}
	120	4.49±0.04^{d}	7.59±0.28^{b}	2.63±0.09^{b}	0.03±0.03	0.09±0.00	0.77±0.03^{c}	8.37±0.87^{b}
H2	7	4.38±0.01^{d}	6.53±0.09^{c}	1.02±0.03^{c}	traces	–	0.50±0.00^{c}	3.12±0.37^{c}
	15	4.35±0.03^{c}	7.21±0.22^{d}	1.15±0.03^{d}	traces	–	0.40±0.03^{ac}	3.75±0.25^{d}
	30	4.30±0.02^{d}	7.96±0.19^{e}	1.24±0.03^{d}	traces	0.09±0.00	0.46±0.03^{a}	3.37±0.25^{e}
	60	4.30±0.01^{d}	7.83±0.22^{c}	1.64±0.06^{d}	–	0.09±0.00	0.68±0.03^{b}	3.25±0.06^{e}
	120	4.35±0.03^{e}	8.20±0.12^{c}	2.04±0.09^{c}	traces	0.09±0.00	0.77±0.03^{c}	6.12±0.56^{c}
G	7	4.87±0.03^{e}	4.40±0.19^{b}	1.33±0.06^{d}	traces	0.09±0.03	0.40±0.06^{abc}	6.81±0.31^{d}
	15	4.89±0.03^{d}	4.83±0.19^{b}	1,80±0.06^{e}	traces	0.12±0.03	0.37±0.03^{abc}	7.75±0.69^{e}
	30	5.00±0.02^{e}	4.83±0.15^{b}	2.45±0.09^{e}	traces	0.09±0.03	0.46±0.03^{a}	9.12±1.00^{f}
	60	4.92±0.02^{a}	4.89±0.09^{a}	3.07±0.15^{b}	0.12±0.03	0.12±0.03	0.50±0.03^{c}	10.06±0.44^{b}
	120	4.88±0.01^{a}	5.20±0.22^{a}	3.56±0.12^{d}	0.12±0.00	0.09±0.03	0.65±0.06^{ac}	14.75±0.69^{a}

a, b, c, d, e, f values marked with different letters within columns differ significantly

C: Control, I: Inoculated, H1: Inoculation + 1.0% hydrolyzed corn meal, H1.5: Inoculation + 1.5% hydrolyzed corn meal, H2: Inoculation + 2.0% hydrolyzed corn meal, G: Goldzyme

Table 3. Changes of WSC content during fermentation

Treatment	WSC content, g/kg alfalfa silage						Fermented WSC on day 120	
	Days of fermentation							
	0	7	15	30	60	120	g	%
C	29.5	4.7	4.1	2.9	2.3	2.3	27.2	92.20
I	29.5	4.3	3.5	2.9	2.2	2.1	27.4.	92.88
H1	35.4	5.1	4.7	4.0	3.5	3.3	32.1	90.68
H1.5	38.3	5.3	5.1	5.1	4.2	4.0	34.3	89.56
H2	41.2	6.6	5.6	5.5	5.5	5.1	36.1	87.62
G	29.5	4.1	3.5	2.9	2.5	2.2	27.3	92.54

C: Control, I: Inoculated, H1: Inoculation + 1.0% hydrolyzed corn meal, H1.5: Inoculation + 1.5% hydrolyzed corn meal, H2: Inoculation + 2.0% hydrolyzed corn meal, G: Goldzyme

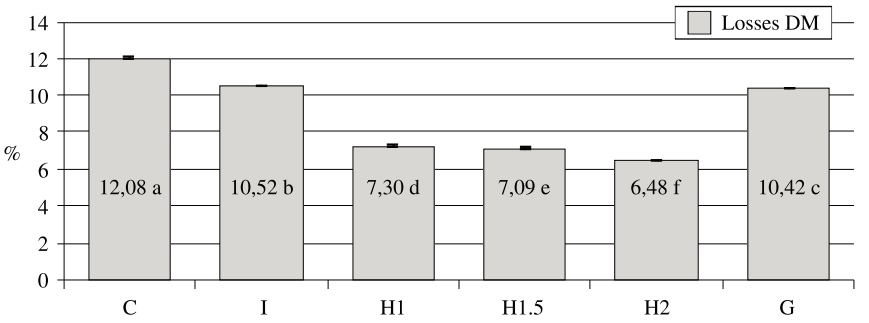

Figure 1. Dry matter loss depending on the application rate of hydrolyzed corn meal

a, b, c, d, e, f values marked with different letters of columns differ significantly
C: Control, I: Inoculated, H1: Inoculation + 1.0% hydrolyzed corn meal,
H1.5: Inoculation + 1.5% hydrolyzed corn meal, H2: Inoculation + 2.0% hydrolyzed corn meal
G: Goldzyme, DM: dry matter

These results can be explained with the rapid increase of the number of homofermentative lactic acid bacteria, restricting the activity of the undesirable species, thus decreasing fermentation losses.

There are several previous reports on the use of carbohydrate sources, mainly molasses, as silage additives, but no any reference could be found in the literature on the use of hydrolyzed corn meal. There are a great number of experimental results showing that added molasses increases lactic acid, decreases acetic acid and pH, inhibits Clostridia and the proteolysis, furthermore substantially decreases organic matter losses in the silage. Despite of these benefits, molasses is only rarely used as an additive because of difficulties in application (*Woolford* 1984). Sugar (used for animal feed) was also used for ensiling (*Weise* 1967, *Gross* 1969). Just as molasses, it is not available on the market at that time. Therefore more than 10% should be used, or a combination with malt for a partial hydrolysis of starch should be used to ensure the necessary amount of sugar for the fermentation (*Rydin* 1963, *Zimmer* 1964).

An argument against carbohydrate additives are that the added sugar is used not only by the lactic acid bacteria, but also by the undesirable flora. *Weise* (1967) found that the carbohydrate additive stimulated not only the LAB but the Clostridia and yeasts, as well. *Hartfiel* and *Marquering* (1968) have shown when adding 10 g with ^{14}C-labelled saccharose to grass silage, that 20% of the sugar was lost in the form of CO_2. Other authors (*Wieringa* 1961, *Ohyama et al.* 1975) reported that the carbohydrate supplement alone does not assure that LAB will be the dominant bacteria in the silo; therefore an inoculation with LAB is beneficial. The findings of *Jones et al.* (1992) support this statement, who found that a sugar supplement of 2% did not influence the final pH of the silage, when adding it to a high DM alfalfa (330–430 g/kg). However a combination of sugar and inoculation significantly decreased the pH, acetate and NH_3-nitrogen and increased lactate concentration.

The additive G resulted in a better silage quality than the C only in the first 30 days of fermentation. Later on the development of acetic acid was positive effected. All variables of G treated silages were inferior in every respect compared to the H1, H1.5 and H2 silages.

The results of ensiling experiments with cell wall degrading enzymes are controversial. This might be due to the different composition and activity of enzymes, produced by various microorganisms. *John* (1991) emphasizes that a substantial hydrolysis of roughage can only be expected when the enzyme complex highly active and has a suitable proportion of endoglucanase, xylanase, and β-glucosidase activity. *Kung et al.* (2003), explain the varying results, by mentioning that the conditions in the silo greatly differ from those necessary for an optimal functioning of cellulolytic enzymes (temperature 50 °C and pH 4.5). He noted that the circumstances, under which the activity of the cellulase preparations is measured, differ from the conditions in the silo. *Kung et al.* (2003) are convinced that the enzyme concentration of the treated products is so small that it is a question whether they may have any effect on the fermentation.

Conclusions

Corn meal hydrolyzed with α-amylase and amyloglucosidase enzymes is a good source of fermentable carbohydrates for LAB and suitable as an additive for controlling lactic acid production in alfalfa fermentation. The addition of H1.0 to alfalfa wilted to 320 g/kg DM is sufficient to achieve a silage of good quality with a favourable lactic:acetic acid ratio. One % of hydrolyzed corn meal substantially reduces the losses in the silo. DM losses were by 7.3%. The difference between the two groups was 4.8% (relative 39.6%) of the control value. The addition of hydrolyzed corn meal and treated with an inoculant decreased the NH_3-content of the silage. Hydrolyzed corn meal supplementation resulted better silage quality and lower fermentation losses than the Goldzyme supplementation.

Lucerna silózása hidrolizált kukoricakiegészítéssel és baktériumos oltással

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Összefoglalás

A szerzők a kukorica keményítőjének α-amilázzal (BAN 480) és amiloglükozidázzal (Spirizyme) történő lebontásával nyert, majd megszárított kukoricát használtak a zöld-lucerna erjedőképességének javítására. A hidrolízis során a kukorica keményítőjének 89%-át bontották le vízoldható szénhidráttá, így a silózáshoz felhasznált kukorica vízoldható

szénhidráttartalma 587,4 g/kg kukorica volt. A hidrolizált kukoricakiegészítés erjedésre gyakorolt hatását modell silózási kísérletben vizsgálták. Ennek során minden kezelésből 25 db 850 ml térfogatú modell silót töltöttek, amelyek közül az erjedés 7., 15., 30., 60. és 120. napján 5–5 silót felbontottak. A 32,3% szárazanyag-tartalomig fonnyasztott lucernához 1,0; 1,5 és 2% hidrolizált kukoricát adagoltak és egyúttal baktériumkultúrával oltást is végeztek. Az oltás élőtelepszáma 105 CFU/g lucerna volt. Egy kezelés esetében baktériumkultúrát és sejtfalbontó enzimeket tartalmazó tartósítószert is vizsgáltak (Goldzyme). Megállapították, hogy a hidrolizált kukoricakiegészítés a dózissal arányosan szignifikánsan növelte a szilázsban a tejsav mennyiségét, és ugyancsak szignifikánsan csökkentette a szilázs pH-ját, ecetsav-, valamint NH_3-tartalmát és mérséklődött a szilázsban a szárazanyag- veszteség is. Az 1,0% hidrolizált kukoricakiegészítéssel már kifogástalan minőségű, kedvező tejsav:ecetsav arányú stabil szilázst sikerült előállítani. A Goldzyme-mel készült szilázs minden lényeges tulajdonság (pH, tejsav-, ecetsav-, NH_3-tartalom) tekintetében szignifikánsan gyengébb minőségű volt a hidrolizált kukoricakiegészítéssel előállított szilázsok minőségénél.

Kulcsszavak: lucerna, szilázs, szénhidrát adalék, baktériumos oltás.

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