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COMBINATION OF ITALIAN RYEGRASS AND WINTER CEREALS PROVIDES NEW ALTERNATIVE FORAGES IN DAIRY NUTRITION

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1. BACKGROUND OF THE STUDY, OBJECTIVES

In recent years, difficulties occurring in corn cultivation (i.e., groundwater shortages, mycotoxin contamination) forced dairy farmers to consider alternative silages. The vegetation period of winter crops covers autumn, winter and early spring, when the soil conserves enough moisture for vegetation up to harvest because of generally higher winter precipitation, and preceding the dry summer period. However, the main vegetation period of corn in temperate zone covers the warmest and driest periods, which can decrease yield of whole plant corn dramatically. Finding acceptable alternative forage to replace whole crop corn silage will be a critical challenge for the success of future dairy operations if climate change induced factors continue to affect corn production, particularly in Europe.

In the present study mixtures of winter cereals and Italian ryegrass plus winter cereal silages was studied with the objective of evaluating the feed value, aroma profile, digestibility, degradability, ruminal fermentation and energy values of different combinations winter cereal as well as Italian ryegrass (*Lolium multiflorum Lam.*) and winter cereal silages. The components of the mixture complement each other's properties: the digestibility of barley, winter oats and Italian ryegrass is excellent, while wheat and triticale give high yields and triticale is an indicator plant for determining harvest date. Triticale also contains perhaps the best quality of fiber fractions with the highest proportion of NDF. This can be crucial in the nutrition of high yielding dairy cows fed high proportion of concentrate to provide enough precursors for milk fat synthesis. Legumes are not included in the present mixture due to its lower fiber digestibility and high buffering capacity than winter cereals or Italian ryegrass. However, the main trouble with legumes, that they contain high proportion of soluble N containing compounds, and increasing the ammonia (NH_3) production in the rumen, which resulting in higher urea concentration in blood decrease the fertility (conception rate) of the cow.

2. MATERIALS AND METHODS

The experiment was conducted in two different phases. In experiment I two mixtures of ensiled Italian ryegrass and winter cereals were studied for chemical composition, fermentation characteristics, microbial counts, ruminal degradability and energy and protein evaluation (digestibility study). In experiment II, four mixtures (two winter cereal based + two Italian ryegrass plus cereal mixtures) were studied for chemical composition, fermentation characteristics, microbial counts, ruminal degradability and ruminal fermentation study. Additionally, subsequent cuts were done to study the nutritional profile of the forage at different crop mixtures from leafy to early heading stage (harvesting stage). The quality of silages was evaluated for aroma profile using electronic – nose technique.

2.1. Experimental site and ensiling procedures (Experiment I)

The trial was carried out on a large-scale farm (Galgamenti Agricultural Limited Company, Tura, Hungary - 47.593637 N, 19.576483 E, at 119 m altitude). Two different forage mixtures (commercial products, producer: *Agroteam S.p.a.*, Torrimpietre (RM), Via di Granaretto, 26, 00054 Italy) were studied: mixture A' (40% of three cultivars of Italian ryegrass + 20% of two cultivars of winter triticale + 20% of two cultivars of winter oats + 15% of winter wheat + 5% of winter barley; and mixture B': 55% of three cultivars Italian ryegrass + 45% of two cultivars of winter oats). Experimental field was 30,600 m^2 (width: 36 m; length: 850 m) for each mixture. Deep loosening and disc plus cylinder cultivation were executed as stubble tillage after winter rapeseed (*Brassica napus L.*). Slurry (10 m^3/ha) and 300 kg/ha artificial fertilizer (NPK: 14:10:20) was applied before sowing on sandy soil. Seedbed was prepared by Kongskilde VibroFlex 7400 cultivator (lifted). The two different forage mixtures were sown on 11th September 2017 (mixture A': 75 kg seed/ha; mixture B': 75 kg seed/ha) with depth of 2-5 cm with John Deere 740 A type seed drill. Plant protection treatment was not applied during the growing season. The annual precipitation was 718 mm in 2017. Cutting was carried out in heading stage of triticale based on the existing extended BBCH-scale (Meier, 2001) [25th April, 2018, BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemical Industry) 57-58], at 10 cm stubble height. After cutting mixture A' (dry matter (DM): 189 g/kg; crude protein (CP): 161 g/kg DM; neutral detergent fiber (NDF): 485 g/kg DM, and total sugar: 137 g/kg DM) and mixture B' (DM: 195 g/kg; CP: 159 g/kg DM; NDF: 519 g/kg DM and total sugar: 138 g/kg DM) were wilted (24h) without any movement on the windrow. The wilted forage was chopped by a forage harvester (John Deere 7300) on concrete surface with theoretical chop length (TCL) of 9 mm (weight: 800 kg of harvested forage). Wilted and chopped material of 510 g were packed by hand into anaerobic glass jars capacity of 0.72 litter (0.00072 m^3 volume, total no. of laboratory silos = 30 (15/mixture), replicated five times) and stored in a controlled laboratory at a temperature of 21 °C. The applied density was 708 kg wilted material/m^3 (mixture A': 200 kg DM/m^3; mixture B': 219 kg DM/m^3).

2.2. Experimental site and ensiling procedure (Experiment II)

The trial was carried out on a medium-scale farm (Hungarian University of Agriculture and Life Sciences, Kaposvár Campus, Kaposvár, Hungary – 46°22' N 17°48' E, 153 m altitude (GeoDatos, 2020). Four different forage mixtures (commercial products, *Agroteam S.p.a.*, Torrimpietre (RM), Via di Granaretto, 26, 00054 Italy) were studied: mixture A (40% of two cultivars of winter triticale + 30% of two cultivars of winter oats + 20% of winter barley + 10% of winter wheat), mixture B (50% of two cultivars of winter triticale + 40% of winter barley + 10% of winter wheat), mixture C (55% of three types of Italian ryegrass + 45%

of two cultivars of winter oat), mixture D (40% of three types of Italian ryegrass + 30% of two cultivars of winter oat + 15% of two cultivars of winter triticale + 10% of winter barley + 5% of winter wheat). The experimental field allotted 3 hectares to each mixture. Deep loosening and disc + cylinder cultivation were executed as stubble tillage. 351 kg/ha artificial fertilizer (NPK: 16:16:16) was applied before sowing. Seedbed was prepared by Kongskilde VibroFlex 7400 cultivator (lifted). The four different forage mixtures were sown on 29th September 2018 (mixture A: 75 kg seed/ha; mixture B: 75 kg seed/ha; mixture C: 75 kg seed/ha, mixture D: 75 kg seed/ha) with depth of 3 cm with John Deere 740 A type seed drill. Plant protection treatment was not applied during the growing period. The annual precipitation was 425 mm in 2018 (World weather online/Kaposvár monthly climate average). Cutting was carried out in the heading stage of triticale based on the existing extended BBCH-scale (Meier, 2001) [4th of May 2019, BBCH (Biologische Bundesanstallt für Land-und Forstwirtschaft) (1997) 51-58. (Italian ryegrass: BBCH51; oat: BBCH51; triticale: BBCH53; winter wheat: BBCH52; winter barley: BBCH58)]. The fresh mixture A (dry matter 186 g/kg; crude protein: 125 g/kg DM; NDF: 566 g/kg DM, total sugar: 168 g/kg DM), mixture B (dry matter 184 g/kg; crude protein: 117 g/kg DM; NDF: 579 g/kg DM, total sugar: 166 g/kg DM), mixture C (dry matter 168 g/kg; crude protein: 108 g/kg DM; NDF: 535 g/kg DM, total sugar: 168 g/kg DM) and mixture D (dry matter 173 g/kg; crude protein: 95 g/kg DM; NDF: 532 g/kg DM, total sugar: 140 g/kg DM) were wilted to 35% DM (24h) without any movement on the windrow. The wilted forage ensiled as described in Experiment I.

2.3. Chemical analysis (Experiment I and II)

Five laboratory silos per experimental mixtures were opened on 7, 14 and 90 days after ensiling (n=15/treatment). DM, CP, CF, neutral detergent fiber (NDF), acid detergent fiber (ADF), EE, ash, and total sugar content of all mixtures were determined. The chemical analyses of the fresh and mixtures silages were done following (AOAC, 2006) protocol and Van Soest et al. (1991) (ADF, NDF, ADL) following sodium sulphite assay. Approximately 25 g composite sample was taken from each laboratory silo immediately after opening. The sample silage was mixed with 100 ml of distilled water. After hydration for 10 min using blender, the diluted material was then filtered through cheese cloth and then pH was determined by using a digital pH meter (Metrohm 744, Switzerland). The lactate was analyzed by high-performance liquid chromatography (HPLC) method developed by Megias et al. (1993). Acetic acid, butyric acid, propionic acid and ethanol were measured by gas chromatography (Chrompack, Model CP 9002, The Netherlands) as described by Playne (1985). Ammonia concentration was determined by a modified Berthelot method (Chaney and Marbach, 1962).

2.4. Microbiological counts (Experiment I and II)

Aerobic mesophilic microorganism count (AMC) or mold and yeasts count of ensiled forage at the three opening days (7, 14 and 90 days) were determined at the Laboratory of Hungarian University of Agriculture and Life Sciences, Kaposvár Campus following the standard laboratory protocols (EN ISO 4833-1:2013 and EN ISO 21527-1:2008) using a standard dispersion plate method (Pitt and Hocking, 2009). Total microbiological counts were expressed as colony forming units per gram (CFU g-1) and were transformed into log10 to obtain the lognormal distribution.

2.5. Aroma profiling with electronic nose (Experiment II)

Sample from fresh green forage and from each opening day (7, 14 and 90) were frozen (n=80, 20/treatment) before it sent to the laboratory of ADEXGO Ltd. in Herceghalom. Frozen samples were thawed and chopped with scissors. The smell fingerprints of the silage samples were acquired in 3 replicates by measuring 3-

times 1 g of each into 20 mL headspace vials which were then sealed with a magnetic cup and a PTFE septum. The EN measurement was performed with a Heracles Neo 300 ultra-fast GC analyzer (Alpha MOS, Toulouse, France), specifically designed for the rapid analysis of volatile compounds. The EN was equipped with a PAL-RSI autosampler unit for standard handling the samples, generating headspace, and injecting the headspace into the Heracles analyzer unit, including an odor concentrator trap and two metal capillary columns (Restek MXT-5: length 10 m, ID 0.18 mm, thickness: 0.40 µm, low-polarity stationary phase composed of cross bond 5% diphenyl / 95% dimethyl polysiloxane; and Restek MXT-1701: length 10 m, ID 0.18 mm, thickness: 0.40 µm, mid-polarity stationary phase composed of cross bond 14% cyanopropylphenyl / 86% dimethyl polysiloxane (Restek, Co., Bellefonte, PA, USA). The volatile compounds were separated by both columns simultaneously and detected with two flame ionization detectors (FID). The autosampler and the analyzer were operated with the software AlphaSoft ver. 16 (Alpha MOS, Toulouse, France), and the same software was used for data acquisition and data transformations. The retention times of the volatiles recorded on both columns were converted to Kováts retention indices (RI) that relate the retention time of the investigated volatile molecules of a sample to the retention time of n-alkanes under the same conditions (Alpha MOS, 2018). The RI characterizes the volatile compounds on the specific columns and can be assigned to specific aroma recorded in the AroChemBase v7 of AlphaSoft software. In this study, "1-A" as an identifier after the RI refers to column MXT-5 and "2-A" refers to column MXT-1701. Before the analysis, a method was created with the following parameters of the PAL-RSI Autosampler and Heracles GC analyzer: Autosampler: incubation at 40 °C for 5 min with 500 rpm agitation to generate headspace, 1 mL of headspace injected into the Heracles analyzer, flushing time between injections: 90 s; Analyzer: carrier gas: hydrogen, the flow of carrier gas: 30 mL/min, trapping temperature: 30 °C, initial oven temperature: 50 °C, the endpoint of oven temperature: 250 °C, heating rate: 2

°C/s, acquisition duration: 110 s, acquisition period: 0.01 s, injection speed: 125 μl/s, cleaning phase: 8 min.

2.6. Ruminal degradability (Experiment I and II)

After the ninety days of fermentation, the ensiled mixtures were subjected to ruminal degradability study. The ruminal degradability trial was carried out with three multiparous non-lactating Holstein-Friesian dairy cows (600±35 kg body weight) previously surgically fitted (ethical permission number - SOI/31/01044 – 3/2017) with a ruminal cannula (10 cm id., Bar-Diamond Inc., Parma, Idaho, USA) at the experimental dairy farm of Hungarian University of Agriculture and Life Sciences, Kaposvár Campus. Cows were fed total mixed ration (TMR) formulated according to the dairy nutrient requirement and feeding standard (NRC, 2001) in equal portions at 8:00 and 14:00 on *ad libitum* basis. The baseline diet [9.12 kg dry matter intake (DMI)/day; 6.32 MJ NE_l /kg DM; 14.4% CP, 39.06% NDF, 23.66% ADF, and 35.71% non-fibrous carbohydrate (NFC)] consisted of 5.5 kg day^{-1} of corn silage, 3.5 kg day^{-1} of alfalfa haylage, 3.5 kg day^{-1} of vetch-triticale haylage, 3 kg day^{-1} of concentrate, 1 kg day^{-1} of grass hay and 0.75 kg day^{-1} of liquid molasses. The cows consumed the daily allotted TMR with no daily feed refusal throughout the course of the experimental period. Water was available *ad libitum*. Rumen incubations were carried out according to Herrera-Saldana et al. (1990). Nylon bags of 5×10 cm with pore size of 53 µm (Ankom, USA) filled with sample weight of 5.0 g (on air dry matter basis) was incubated for 0, 2, 4, 8, 16, 24, 48 and 72 h incubation times. In each incubation, 60 bags per sample were used (5 bags × 4 replications per sample × 3 cows). The 0 h samples were not placed in the rumen, but they were soaked and rinsed as described below. Removed bags were placed in cold tap water immediately after removal from the rumen, and they were washed by hand until the water was clear. After washing, the bags were dried in a forced air oven at 60 °C for 48 h, air equilibrated and weighed. Residues from the bags were pooled within time and animal, finely ground by mortar and pestle to pass through a 1-mm screen and retained in sealed containers to determine DM, CP, NDF and ADF. Feeds were analyzed for nitrogen according to Kjeldahl (AOAC, 2006), and thereafter, CP was determined by the total nitrogen (N) × 6.25. The NDF and ADF contents were residual portions after rinsing according to Van Soest et al. (1991).

2.6.1. Calculations and statistical analysis
2.6.1.1. Calculations

Residues from the nylon bags at each incubation time were analyzed for DM, CP, NDF and ADF as described above. Ruminal nutrient disappearance data were used to determine nutrient degradation parameters using the equation (Ørskov and McDonald 1979):

$$P = a + b\,(1 - e^{-ct}),$$

where *P* is the DM, CP, NDF or ADF disappearance (%) at time *t*, *a* is the soluble fraction (%), *b* is the potentially degradable fraction (%), and *c* is the rate of degradation of the *b* fraction (%/h). Effective degradability (ED) of DM, CP, NDF and ADF was then calculated according to the equation (Ørskov and McDonald 1979):

$$ED = a + ((b \times c)/(k + c)),$$

where *k* is the rumen outflow rate assumed to be 1, 5 and 8%/h and *a*, *b*, and *c* are as described above. NLIN program in SAS (version 9.4; SAS Institute, Inc., Cary, NC, USA) was used to calculate the values of *a*, *b*, and *c*.

2.7. Ruminal fermentation (Experiment II)

The ruminal fermentation trial was carried out with three multiparous non-lactating rumen cannulated Holstein-Friesian dairy cows. The ruminal fermentation trial was conducted with mixtures silage following ninety days fermentation in the form of baleage capacity (578 – 675 kg) for all ensiled mixtures. Cows consumed TMR (control diet) as described in ruminal degradability (see *chapter 2.6*) plus ensiled mixtures by substitution 3.5 kg day^{-1} (experimental diet 1, 2, 3 and 4), instead of vetch-triticale haylage. The daily ration of both the control and experimental diets were given in 2 instalments (8:00 am and 14:00 pm). The pre-feeding period lasted 14 days, which was followed by the 14-day experimental phase. Rumen fluid sampling was performed twice a week (Monday and Wednesday). On sampling days, approx. 150 ml of rumen fluid samples were collected 3 times a day (immediately before morning feeding and then 3 and 6 hours thereafter, n=36/mixture) through the cannula using ruminal fluid collection device (Bar-Diamond Inc., Parma, Idaho, USA). The pH was measured immediately using a digital pH meter (Metrohm 744, Switzerland). Ammonia was determined by Berthelot method (Chaney and Marbach, 1962). Thereafter samples were centrifuged to analyse the volatile fatty acid (VFA) and lactic acid. The concentration of volatile fatty acids (acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid or n-valeric acid, i-caproic acid, caproic acid) of rumen fluid and silages were measured by gas chromatography (Chrompack, Model CP 9002, The Netherlands) as described by (Playne, 1985). The lactate was analyzed by high-performance liquid chromatography (HPLC) method developed by (Playne, 1985).

2.8. Energy and Protein Evaluation

Digestibility was studied at the National Agricultural Research and Innovation Centre Research, Herceghalom, Hungary using mixture A' silage (Experiment I). Six wethers Hungarian Merino sheep (4 years of age) with an average body weight of 84.56±5.53 kg was housed in individual metabolic cages with slatted floors with *ad libitum* access to water. The trial consisted of a 10-days adjustment period followed by 5-days of complete faeces collection. The experimental ensiled mixture was offered as the sole feed to the sheep and fed two equal meals per day (0700 and 1500 h). A daily ration was determined on the basis of live weight (above maintenance DM requirement, approximately 2% of the body weight). The sheep received 1.405 kg dry matter intake (DMI)/day plus 30 g mineral and vitamin premix (producer: Bábolna Takarmányipari Ltd, Nagyigmánd, Hungary) plus 10 g NaCl daily. Feed intake, feed refusal and faecal output were recorded daily during the collection period. A 25% sample of faeces was sub-sampled daily from each animal, dried in a forced air oven at 60°C for 24 h and ground through a 1 mm screen to determine the DM percentage. Feed samples were taken at the beginning of adaptation period, and at the beginning and the end of collection period. Feed and faecal samples were analysed for DM, CP, CF, EE, ash, NDF, ADF were determined according to the official methods in Hungary (Hungarian Feed Codex, 2004).

2.8.2. Calculation of digestibility, energy and protein values

2.8.2.1. Digestibility

The digestibility coefficient (DC, %) for nutrients was calculated for each animal on the basis of quantitative data for intake and output according to the classical formula: DC (%) = 100 × (NI-NE)/NI, where, NI represented the nutrient intake and NE expressed the nutrient excreted.

2.8.2.2. Energy evaluation

The net energy for lactation, maintenance and growth was calculated on the basis of digestible nutrients as suggested by the NRC (2001).

2.8.2.3. Protein evaluation

The protein evaluation was done following the Hungarian metabolizable protein system for ruminants (Schmidt et al., 1998). The formulas proposed for the calculation of protein values of feed was the follows:

MPE g/kg DM = 0.9 × (UDP - ADIN × 6.25) + 160 × FOM × 0.8 × 0.8

MPN g/kg DM = 0.9 × (UDP - ADIN × 6.25) + RDP × 0.9 × 0.8 × 0.8.

where MPE energy dependent metabolizable protein and MPN – Nitrogen dependent metabolizable protein UDP – Rumen undegradable protein, ADIN – acid detergent insoluble nitrogen, RDP – Rumen degradable protein, FOM – Fermentable organic matter, FOM = DOM – (UDP + digestible fat + fermentation products + bypass starch), where DOM – Digestible organic matter.

2.9. Statistical analysis

2.9.1. Nutritional composition, fermentation characteristics and microbiological count

Data were analysed using the GLM procedure for ANOVA in SAS 9.1 software (SAS Inst. Inc., Cary, North Carolina, USA). Significant mean value differences were evaluated by Tukey's test following a post hoc comparison of means. A significance level of $P<0.05$ was used.

Variables for nutritional composition, fermentation characteristics and microbiological count among the three opening days, different crop mixtures and their interaction was computed using the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ij}$$

where Y_i is the observation in the i^{th} different opening days, j^{th} crop mixture and their interaction, μ is the overall mean, α_i is the i^{th} opening days effect, β_j is j^{th}

crop mixture effect, γ_{ij} is the interaction of opening days and crop mixture and ε_{ij} is the random error.

2.9.2. Digestibility, in situ degradability and ruminal fermentation

Comparison of means for degradability components were performed following model;

$$Y_i = \mu + \alpha_i + \varepsilon_i,$$

where Y_i is the observation in the i^{th} silage type, μ is the overall mean, α_i is the i^{th} silage type effect and ε_i is the random error. Comparison of means for effective nutrient degradability was computed for 1%, 5% and 8% rumen outflow rates. Comparison of the means of variables between treatments for rumen fermentation characteristics was computed using two-way ANOVA and following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where Y_{ij} is the observation in the i^{th} treatment and j^{th} rumen fluid sampling period; μ is the overall mean; α_i and β_j is the i^{th} treatment and the j^{th} rumen fluid sampling period effects and; ε_{ij} were the random errors.

2.10.3. Multivariate data analysis

The multivariate data of the EN measurements describing the odor profiles of the feed samples were analyzed with the AlphaSoft (ver. 16) software. The chromatograms were transformed into a series of variables called sensors based on the identified chromatogram peaks (Kovacs et al., 2020). The name of a sensor originated from the location of the peak within the chromatogram and was identical to the respective RI. The intensity of the sensor was calculated from the area under the respective chromatogram peak. Principal component analysis (PCA) was performed using the sensor data to detect outlier records and to describe the non-supervised clustering of the samples within the multidimensional space defined by the sensor variables (Naes et al., 2002). The PCA models were characterized by the discrimination index (%) between the classified groups, where positive values indicated group separations without overlapping on the examined plane of principal components. Supervised classification models were built using linear discriminant analysis (LDA) to find linear combinations of the sensor variables, optimally discriminating against the pre-defined groups (Naes et al., 2002). The accuracy of the LDA classification models was tested with leave-one-out cross-validation, when a single record was left out of the modelling process and was used for testing by predicting its group identity – the process was repeated iteratively until all samples were used for validation once (Naes et al., 2002). The cross-validations were evaluated based on the validation score, representing the ratio of correctly classified samples. The sensor selection function of AlphaSoft was used for tracing the most distinctive variables that show the largest capability to contribute to an LDA model identifying the actual pre-defined groups. The LDA calculations based on the selected sensors were also performed, and the impact of the sensors was evaluated by their orientations in PCA and LDA bi-plots. The volatile compounds described by the selected sensors were identified using the AroChemBase database.

3. RESULTS (Experiment I)

3.1. Nutritional composition of ensiled Mixtures

The DM content of ensiled mixtures did not change greatly up to 90 days of fermentation, because the laboratory silos were in anaerobic conditions and had no seepage loss. For both ensiled mixtures, CP was well preserved attributed to lactic acid type fermentation. Well-preserved CP can improve the ruminal degradability of nutrients particularly fiber degradability (NDF and ADF) as well as improve protein dependent metabolizable energy (MPN) utilization. The high CP value is a direct reflection of the quality of the present mixtures at the time of harvest (early heading stage) before ensiling as well as higher proportion of Italian ryegrass than cereal forage in the total mixed ensiled forage because Italian ryegrass has more protein than cereals. After the end of fermentation, the ensiled mixture had moderate NDF and ADF contents.

3.2. Fermentation characteristics of ensiled Mixtures

The rate and extent of reduction in pH was continuous within 90 days of ensiling for both silage mixtures. The rapid decrease in pH prevents breakdown of plant proteins and helps to inhibit growth of spoilage microbes. The reduction of pH was mainly caused by a rapid and intensive production of LA. Fermentation products were limited to three principal products: lactic acid (LA), acetic acid (AA), and ethanol. Other volatile fatty acids (VFAs) like propionic acid (PA), butyric acid (BA), valeric acid (VA) and caproic acid (CA) were below detectable concentrations (<0.1 g/kg of DM). Mixture A' had higher lactate, acetate and ethanol contents than mixture B'. However, there was no difference ($p>0.05$) in LA/AA, LA (%TFA) and NH_3 – N contents between mixture silages. During the 90 days of fermentation, LA continued to be the major fermentation product resulting in a high LA/AA ratio over the storage periods.

3.3. Microbiological quality

The microbiological quality result indicates that the mold and yeast count (Log_{10} CFU g^{-1}) was low and there was no negative report on fermentation end products associated with mold and yeast count.

3.4. Ruminal degradability

The potential ruminal degradability for DM of mixture A' and B' silages was 39.41% and 39.16%, respectively and the effective rumen DM degradability at 8% rumen outflow rate (ED_8) of mixture A' and mixture B' were 48.47% and 48.23% respectively. The potential ruminal degradability of CP for mixture A' and B' silages was 43.59% and 31.87% respectively and the effective rumen CP degradability at 8% rumen outflow rate (ED_8) of mixture A' and mixture B' were 67.27% and 67.04% respectively. The low DM and CP degradability could be attributed to the inclusion of cereals. On the other hand, ensiling could also affect the CP degradability as compared to fresh green forage. The potentially degradable NDF fraction, its degradation rate and effective ruminal degradability-8 (ED_8) were 80.23%. 0.017, and 18.68% (mixture A') and 94.35%, 0.014 and 16.37% (mixture B'). Potentially degradable ADF fraction, its rate of degradation and effective ruminal degradability (ED_8) were 85.18%. 0.017, and 19.45% (mixture A') and 87.26%, 0.014 and 16.44% (mixture B'). Amount and ruminal degradability of NDF is a very important factor in the dairy cow's nutrition because forage NDF varies widely in its degradability in the rumen and NDF digestibility influences animal performance. The high potential *in situ* NDF and ADF degradability of the present silage mixtures could be associated with crop mixtures, those cultivars selected for high ADF and NDF degradability. The high potential and effective ruminal degradability could be associated with agronomic practices such as early harvesting as well as harvesting from spring growth.

3.5. Energy and protein evaluation

3.5.1. Dry matter and nutrient digestibility

The apparent digestibility of nutrients in the present study was above 67% attributed to the complement effect of ensiled materials as digestibility of barley, winter oats and Italian ryegrass is excellent.

3.5.2. Energy concentration in ensiled mixture

The value for net energy for lactation (NE_l), net energy for maintenance (NE_m) and net energy for growth (NE_g) are all better than the values for alfalfa silage (reported in NRC, 2001) and also exceeds the values of good quality grass silage. Thus, early heading harvest of the mixture was not accompanied by a decline in energy content. Ensiled mixture silage contains very low amount of starch since it harvested at early heading stage before the cereal onset starch such as early dough stage, but the digestibility of the fiber was so favourable that its net energy content averaged 0.9 MJ/kg DM still exceeded the average energy content of cereal silages and appears to be similar to rye silage; however, the value was lower than the energy content of corn silage (reported in NRC, 2001).

3.5.3. Protein evaluation

Both the nitrogen and energy dependent metabolizable protein values were higher than 88 g/kg DM. When a ration is formulated a protein balance (MPN-MPE, g) in the rumen should be also calculated. The nitrogen dependent metabolizable energy value was greater than the energy dependent metabolizable energy value implies that there are more nitrogen/protein as a source of energy than energy for the rumen microbes.

4. RESULTS (Experiment I and II)

4.1. Nutritional composition

4.1.1. Nutritional composition of ensiled mixtures

At the end of 90 days fermentation, the CP contents of Italian ryegrass plus winter cereal silage (mixture C and D) was higher. The high CP value is a direct reflection of the quality of the present mixtures at the time of harvest (early heading stage) before ensiling. The higher proportion of Italian ryegrass than cereal grain (mixture C and D) also resulted higher CP since Italian ryegrass has more protein than cereals.

4.2. Fermentation characteristics

Ensiling affected most of the fermentation characteristics of all ensiled mixtures except mixture C (only pH is affected). The pH values decreased ($p<0.05$) in all ensiled mixtures, however at the end of 90 days fermentation the pH content of ensiled mixtures (except mixture B) were higher. The high pH at day 90 could be associated with low lactic acid concentration probably caused by restricted fermentation which result in low acidification and higher ethanol concentration.

4.3. Microbiological quality

The mold and yeast count (Log_{10} CFU/g) at each opening day were higher than the limit recommended as a quality standard for animal feeds (3.00 (Log_{10} CFU/g) or 1×10^4 (CFU/g) (GMP, 2008) as a result the fermentation process and end products of ensiled mixtures was affected.

4.4. Ruminal degradability

The ensiled mixtures had high effective degradable DM and CP at the three rumen outflow rates (ED_1, ED_5 and ED_8) and moderate potentially degradable DM and

CP. The *in situ* degradability of the examined nutrient content (DM, CP, NDF, ADF) of the mixtures varied greatly depending on the proportion of cereals (mixtures A and B) and Italian ryegrass (mixtures C and D). The degradable fraction of DM and CP in the novel mixtures showed significantly different degradation values depending on whether 45% oats were associated with 40% Italian ryegrass (mixture C) or other cereals (15% triticale, 30% oats, 10% barley, 10% wheat) with 55% Italian ryegrass (mixture D). Significant difference was found in the effective degradability (ED_5, ED_8) of the NDF content of the two Italian ryegrass plus winter cereal silages (mixture C vs. mixture D).

4.5. Rumen fermentation

The replacement of ensiled mixtures with vetch-triticale haylage in TMR did not modify the rumen fermentation characteristics; there was no variation ($p > 0.05$) between control and experimental diets, even the inclusion of 40 – 55% Italian ryegrass (Mixture C and D) did not cause variation. This result also indicated that the inclusion level can be increased beyond this level in a total mixed ration formulation as far as an appropriate forage to concentrate ratio as well as energy requirement of dairy cows maintained depending its production status.

4.6. Aroma profiling

Both the opening days and the mixture types cause reproducible odor differences in the ensiled mixtures. The ensiled mixtures both at day 0 and different opening days, expressed its richness in VOCs at different retention indexes. Most of those VOCs was dominated by esters and have esterification potential compounds which give pleasant odor outcomes consequently contribute to silage flavour due to their volatility. Ethyl esters which is a product of an ester formed from volatile fatty acids and ethanol are the most abundant esters in many silages was reported in the present ensiled mixtures due to the presence of desirable VFA and ethanol. These volatiles would likely produce pleasant fruity odors which has weak negative effect on silage intake. However, some off odor compounds like 3-methylbutanoic acid also called beta-methylbutyric acid, a branched chain alkyl carboxylic and 2-methyl-2-propanol, a simple alcohol with unpleasant camphor-like odor found in fermented mixture C may likely reduce its intake.

5. CONCLUSIONS AND RECOMMONDATIONS

In experiment I**,** the ensiled Italian ryegrass and winter cereals mixtures produces silage with an excellent feed characteristic, digestibility, degradability and energy contents.

In experiment II, the fermentation process underwent lactic acid fermentation type for all ensiled mixtures. However, restricted fermentation affected the output of some fermentation end products with lower lactate production as a result pH was not dropped as rapid as possible. The ensiled mixtures both at day 0 and different opening days, expressed its richness in VOCs at different retention indices. Most of those VOCs was dominated by esters and have esterification potential compounds which give pleasant odor outcomes consequently contribute to silage flavor due to their volatility.

6. NEW SCIENTIFIC RESULTS

1. Nutritive values ($NE_{m,}$ NE_{l}, NE_{g} MPN and MPE) in the combination of Italian ryegrass and winter cereals (triticale oat, wheat and barley) silages tested in our experiments were higher than the values reported for these silages alone in NRC (2001).
2. The detailed analysis of chemical composition, nutritive value and fermentation characteristics of silages of Italian ryegrass and winter cereals in comparison with winter cereal mixtures support that this feedstuff can be successfully included in the ruminant feeding. According to the results of *in vivo* digestibility trial Italian ryegrass and winter cereal mixture (associated with harvesting at early heading stage) has high digestibility and nutritive value and would be a good option for ruminant.
3. An inclusion of about 40-45% of Italian ryegrass causes higher effective degradability of dry matter and crude protein than cereal crop mixture silages alone tested at 8 % of rumen content flow rate.
4. Inclusion of winter cereal mixtures and winter cereals and Italian ryegrass mixtures in dairy cow ration had no effect on rumen fermentation parameters.
5. The opening days and the mixture types of silages cause reproducible odour differences in the ensiled mixtures. Silages made from different combination mixtures of Italian ryegrass and winter cereals expresses richness in volatile organic compounds (VOCs) at different retention indexes. Most of those VOCs were dominated by esters and have esterification potential which give pleasant odour outcomes consequently contribute to silage flavour due to their volatility. As demonstrated by these results, the applied electronic nose technology is a useful tool to describe the quality of ensiled forages. The technology may be used in practical applications to identify defects or preferred smell for certain reasons.

7. SCIENTIFIC PAPERS ON THE SUBJECT OF THE DISSERTATION

7.1. Peer-reviewed papers published in foreign scientific journals

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, Kacsala, L., Drew V., Tóth, T. (2021). Novel mixtures of Italian ryegrass and winter cereals: influence of ensiling on nutritional composition, fermentation characteristics, microbial counts and ruminal degradability. Italian Journal of Animal Science. Vol. 20 (1). https://doi.org/10.1080/1828051X.2021.1924883

Alemayehu, W., Tóth, T., Orosz, Sz., Fébel, H., Kacsala, L., Húth, B., Hoffmann, R., Yakubu, H.G., Bazar, G., Tóthi, R. (2021). Aroma Profile, Microbial and Chemical Quality of Ensiled Green Forages Mixtures of Winter Cereals and Italian Ryegrass. *Agriculture* *11*, 512. https://doi.org/10.3390/agriculture11060512

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Kacsala, L., Húth, B., Hoffmann, R., Tóth, T. (2020): In situ ruminal degradability and fermentation characteristics of novel mixtures of winter cereals and Italian ryegrass plus winter cereal grain silages. Czech J. Anim. Sci., 66 (2021): 302-314. https://doi.org/10.17221/12/2021-CJAS

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Kacsala, L., Bazar, G., Tóth, T. (2019). Nutrient content and fermentation characteristics of ensiled Italian ryegrass and winter cereal mixtures for dairy cows. Krmiva. 61(1). https://doi.org/10.33128/k.61.1.6

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Tossenberger, J., Húth, B., Tóth, T. (2020). Nutritive value of ensiled Italian ryegrass and winter cereal mixture. Acta Fytotechnica et Zootechnica. Vol. 23 (2020). https://doi.org/10.15414/afz.2020.23.mi-fpap.7-14

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Tóth, T. (2020). Effect of climate change on global corn production, its impact on corn silage production and ensiled Italian ryegrass (*Lolium multiflorum Lam.*) and winter cereals mixtures as viable alternative options (*In press: Ethiopian Journal of Animal production*)

7.2. Peer-reviewed papers published in Hungarian scientific journals in English

Alemayehu, W., Tóth, T., Orosz, Sz., Tóthi, R. (2019). Potential forage resources as alternatives to partial or total substitution of corn silage in dairy cattle nutrition: A review. Állattenyésztés és Takarmányozás. 68(2):109-127.

7.3. Proceedings in English

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Kacsala, L., Bazar, G., Tóth, T. (2019). Nutrient content and fermentation characteristics of ensiled Italian ryegrass and winter cereal mixtures for dairy cows. Proceeding of the 26th international conference. June 5 – 7, 2019. Opatija, Croatia. p 28.

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Tóth, T. (2019). Prediction of nutritive value in mixtures of Italian ryegrass (*Lolium multiforum Lam*.) and winter cereals silages: nutrient digestibility and energy contretations using nutrient compositions and fermentation characteristics. In: Tatjana, Čeh; Stanko, Kapun (eds.) Abstract of the proceedings of the 28th international symposium on nutrition of farm animals Zadravec-Erjavec Days 2019. Radenci, Slovenia, (2019) pp. 13-13., 1 p.

7.4. Abstract in English

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Tóth, T. (2019). Effect of climate change on global corn production, its impact on corn silage production and ensiled Italian ryegrass (*Lolium multiflorum Lam*.) and winter cereals mixtures as viable alternative options. The 27th conference of the Ethiopian Society of Animal Production, Addis Ababa Ethiopia.

Alemayehu, W., Tóthi, R., Orosz, Sz., Fébel, H., Tossenberger, J., Húth, B., Tóth, T. (2020). Nutritive value of ensiled Italian ryegrass and winter cereal mixture. The 28th international conference of Animal Science Days, Padova, Italy.

8. OTHER PUBLICATIONS

8.1. Peer-reviewed papers published in foreign scientific journals

Alemayehu, W., Getachew A., Mengistu U. (2015). Supplementing rice bran, Sesbania (*Sesbania sesban*) leaf and their mixtures on digestibility and performance of Kafka sheep fed native grass hay. Int. J Agric Sci. Res. 4(3): 57 – 66.

Worku, A. (2016). Moringa oleifera as a potential feed for livestock and aquaculture industry. African J. of Agri. Sci. and Tech. 4(4): 666 – 676.

Worku, A. (2016). Community based participatory range land management and its benefits in pastoral areas of Ethiopia. African J. of Agri. Sci. and Tech. (AJAST). 4(4): 659 – 665.

8.2. Peer-reviewed papers published in Hungarian scientific journals and magazines

Tóth, T., Alemayehu, W.B., Orosz, Sz., Fébel, H., Tóthi, R. (2019). A klímaváltozás kapcsán előtérbe kerülő újszerű erjeszthető tömegtakarmányok jellemzői (gabona-olaszperje keverékek). Agro Napló. 23 (4). 77-78. pp.

Bekő, D., Orosz, Sz., Tóth, T., Tóthi, R., Alemayehu, W., Fébel, H. (2019). A Dakota gabona-fű keverékszilázs fehérjetartalma: Nettóenergia- és metabolizálható fehérjetartalma *in vivo* kísérleti adatok alapján. Partnertájékoztató hírlevél 19: 10 pp. 18-23., 6 p.

Alemayehu, W., Tóthi, R.; Orosz, S., Fébel, H., Húth, B., Tóth, T. (2020). Olaszperje-gabona keverékszilázs takarmányozási értékének vizsgálata hazai és nemzetközi energiaértékelési adatok alapján. Agro Napló 24: 7 pp. 67-68., 2 p.

Alemayehu, W., Tóthi, R., Orosz, S., Fébel, H., Húth, B., Tóth, T. (2021). Modellvizsgálati adatok az olaszperje-gabona és gabona keverékszilázsok táplálóanyag-tartalmának bendőbeli lebomlásáról. Agro Napló 25: 3 pp. 85-86., 2 p.

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