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Effects of Hybrid and Grain Maturity Stage on the Ruminal Degradation and the Nutritive Value of Maize Forage for Silage

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Abstract: The study aimed to examine the effects of harvesting three maize hybrids at different maturity stages on the ruminal fermentation kinetics, fermentation end-products (volatile fatty acid, ammonia and methane) output, and digestibility of maize plant fractions, as well as the degradability of the resulting silage. Three hybrids were compared (Maximus VIP3, Defender VIP and Feroz VIP) harvested at three grain maturity stages (milk (R3), dough (R4) and dent (R5) grain), while silage samples were collected only at the dent grain stage (R5). Grain digestibility tended to decrease as the maturity stage progressed ($p < 0.05$), while the whole-plant digestibility increased with increasing maturity for the three evaluated hybrids ($p < 0.05$). The gas production of whole-plant at 24 h of incubation was higher for the Maximus hybrid than for the others ($p < 0.05$), with average values of 188, 196 and 207 mL g^{-1} dry matter at stages R3, R4 and R5, respectively. For the in situ ruminal degradation kinetics of silage, instantly degradable dry matter and fiber potential degradability were greater with Maximus maize forage than with the other two hybrids. From the perspective of in vitro results, the Maximus VIP3 hybrid seems to be the most suitable for silage production when harvested between the dough and the dent grain stage of maturity.

Keywords: maize; silage; rumen; fermentation kinetics; in vitro digestibility; methane; Milk2006

1. Introduction

Maize silage is a key feedstuff in the ruminant diets due to the yield of forage biomass and the energy content of the feed [1]. Usually, the dry matter yield and the amount of grain present in the plant were considered the main indicators for the selection of hybrids. However, recent studies have linked several other attributes to the nutritional quality of silage, with emphasis on the use of the vegetative fraction of the plant [2,3]. Di Marco et al. [4] underlined that under unfavorable weather conditions, silage digestibility may be better correlated with cell wall digestibility than with starch content.

The maturity stage at harvest is also one of the likely nutritional quality determinants, and some authors have considered it the most influential factor affecting silage digestibility [4]. In a review, Khan et al. [1] reported a large variability between harvesting stages, being strongly correlated with changes in silage chemical composition, so that early harvesting is related to a lower energy content of silage due to small accretion of starch in the grain, in addition to effluent losses [5]. It is known that as grain matures there is an increase in starch in the grains, whereas nitrogen compounds in leaves are

replaced with fiber and lignin, and the fiber concentration, degradability and composition can limit the extent of digestion [6] and affect methane production in the rumen.

Many factors affect degradability and the products (volatile fatty acids, ammonia, methane, etc.) resulting from fermentation in the rumen, of which both maize hybrid and grain maturity have great notoriety. In vitro and in situ techniques are useful for the study of ruminal fermentation processes under controlled conditions, where substrates are incubated in batch cultures of mixed ruminal microorganisms, and the fermentation end products accumulated during the process can be measured after a certain incubation time [7]. Such laboratory methods are necessary for providing information and obtaining accurate predictions of forage quality quickly and on a large scale.

The hybrids evaluated in this study are considered suitable feedingstuffs for silage production, but data about these materials are still scarce. Our objective was to compare the effects of harvesting three maize hybrids at three different maturity stages on the ruminal fermentation kinetics, production of volatile fatty acids, ammonia and methane production, and digestibility of plant fractions, as well as silage degradability.

2. Materials and Methods

Maize was planted in a tillage land belonging to the Center of the Agrarian and Environmental Sciences of the State University of Middle West (UNICENTRO) in Guarapuava, Paraná, Brazil, with geographic coordinates of 25°23′36′′ S and 51°27′19′′ W and an altitude of 1120 m. The climate of the region is temperate altitude, Cfb (humid mesothermal subtropical), with no dry season, cool summers and moderate winters according to the Köppen classification. Figure 1 shows the average sunshine (hours daily), rainfall (mm), maximum and minimum temperature (°C), and relative humidity (%) during the experimental period, from October 2017 to March 2018.

Maize was planted in the first half of October (mid-Spring) under a no-tillage system. In sowing, row spacing was 0.5 m, the seeding depth was 4 cm and the seed distribution per linear meter was calculated for an estimated final density of 65,000 plants ha^{-1}. The base fertilization consisted of 500 kg ha^{-1} of 08-20-20 (N-P_2O_5-K_2O) fertilizer, and 400 kg ha^{-1} of urea was used at 120 day after planting (DAP).

Sowing followed conventional technical recommendations. The three hybrids were evaluated at three different grain maturity stages, namely milk (soft) grain (stage R3, harvest at 110 to 115 DAP, 10–15 January), dough grain (stage R4, harvest at 120 to 130 DAP, 20–30 January) and dent (hard) grain (stage R5, harvest at 140 to 155 DAP, 10–25 February), with different DAP for each hybrid.

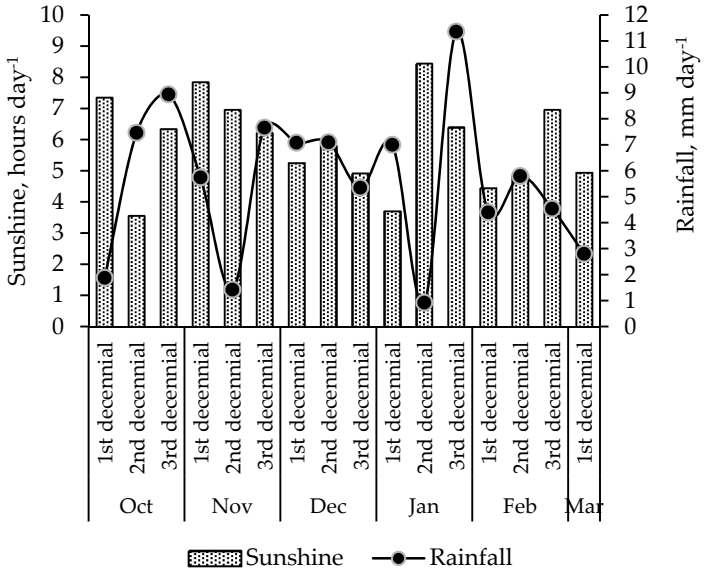

Figure 1. *Cont.*

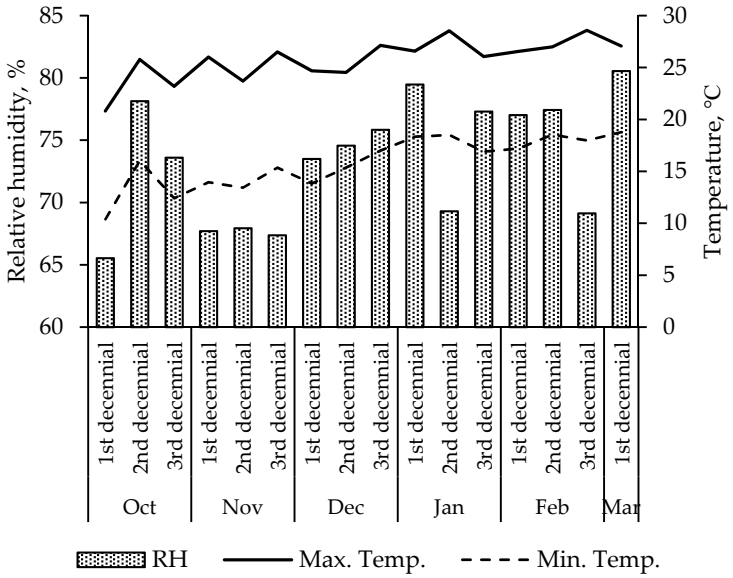

Figure 1. Climograph of the cultivation area plotting sunshine hours, rainfall, maximum and minimum temperature, and relative humidity of air, during the maize growing period (Source: Experimental Station of SIMEPAR / UNICENTRO, Guarapuava, Paraná, Brazil).

2.1. Experiment 1

2.1.1. Materials and Experimental Design

Three insect-resistant maize hybrids with genes to produce vegetative insecticidal proteins (VIP) and with varying silage production capacity were used: Maximus VIP3, Defender VIP and Feroz VIP (Syngenta Crop Protections, Basel, Switzerland), which were single, triple and double hybrids, respectively, and all with a maturity cycle Food and Agriculture Organization (FAO) units of 400. For simplicity, the term VIP will be omitted, and only the main name of each hybrid (Maximus, Defender or Feroz) will be used. A randomized block design was used in a 3 × 3 factorial scheme (three hybrids and three grain maturity stages), with three replications (plots) each. Thus, 27 experimental plots were set up (one for each combination of hybrid and maturity stage), each measuring 7 × 100 m and with 4,550 plants approximately. Each plot was sampled in duplicate, resulting in six samples (3 plots × 2 samples per plot) per experimental treatment. A coefficient of variation of up to 5% was accepted between duplicate samples from each plot. Values obtained for these duplicate samples were averaged for statistical analyses.

2.1.2. In Vitro Incubations

The in vitro studies were performed at the Department of Animal Production of the University of León (ULE) and at the Mountain Livestock Institute (IGM) of the Higher Council for Scientific Research (CSIC) in León, Castilla y León, Spain.

In vitro dry matter digestibility (IVDMD) was determined by two methodologies, the first one adopting the method proposed by Goering & Van Soest [8] and the second following the technique described by Tilley & Terry [9] as modified by Holden [10].

For ruminal fluid collection, three adult sheep with a permanent ruminal cannula were used. The animals were kept in a collective stall with access to water and alfalfa hay ad libitum. For the determination of IVDMD by the technique of Goering & Van Soest [8], samples were ground to 1 mm, and 0.5 g was weighed in duplicate in polyester filter bags with dimensions of 4.5 × 5.5 cm and a mesh size of 25 µm (ANKOM® F57 filter bags, ANKOM Technology, Macedon, NY, USA). Once filled, bags were heat-sealed and placed in specific jars in a DAISY II incubator (Ankom Technology Corp.,

Macedon, NY, USA). Each jar accommodated 27 bags, including one blank and a sample of alfalfa hay that was used as a standard substrate. Each jar contained 1.6 L of culture medium (380 mL of a buffer solution (35 g $NaHCO_3$ + 4 g NH_4HCO_3 per L), 380 mL of a macromineral solution (5.7 g Na_2HPO_4 + 6.2 g KH_2PO_4 + 0.6 $MgSO_4 \times 7H_2O$ per L), 2 mL of a micromineral solution, 2 mL of resazurin, and 756 mL of distilled water) with a final pH of 6.8 and was heated to 39 °C. After reducing the medium (adding 80 mL of a reducing solution (6.25 g Na_2S + 6.25 cysteine-HCl + 40 mL 1N NaOH per L), 400 mL of filtered ruminal fluid was added with constant CO_2 bubbling. The jars were incubated and maintained for up to 48 h at 39 ± 0.5 °C under continuous rotation. After incubation, the bags were removed, washed in water, washed in neutral detergent as proposed by Van Soest et al. [11], dried in a forced ventilation oven at 55 °C for 48 h, and then weighed.

For the determination of IVDMD by the Tilley & Terry technique [9], the same procedures described above were adopted until the end of incubation. After this step, the contents of the jars were drained, and 2 L of acid pepsin solution (2 g pepsin (1:10,000) + 8.35 mL 37% HCl per L) was added to each jar, returning them to the incubator for an additional 24 h incubation. After this time, the bags were removed and washed in water, dried in a forced air oven at 55 °C for 48 h and then weighed.

Two in vitro digestibility studies were completed. In the first one, the Goering & Van Soest [8] method was used to determine the IVDMD of six maize plant parts, namely stem, leaf, husk, cob, grain, and whole-plant. In this case, samples of each fraction were first incubated in diluted rumen fluid for 48 h, and the residue was extracted with neutral detergent. In the second study, the IVDMD of only corn grain and maize whole-plant was assessed by both techniques, Goering & Van Soest [8] and Tilley & Terry [9], and the duration of the incubation in diluted rumen fluid was adapted to the expected residence time of each feedstuff in the rumen. Thus, for grain the first incubation in rumen fluid lasted 16 h, and for the whole-plant the incubation was extended for 24 h.

2.1.3. Fermentation Kinetics

In vitro gas production was performed using a pressure transducer (DeltaOhm, Caselle di Selvazzano, Italy) as described by Theodorou et al. [12], in which 0.5 g duplicate samples were incubated in vials with a capacity of 120 mL containing 50 mL of inoculum (40 mL of culture medium as described above + 10 mL of rumen fluid). Vials containing only inoculum were used for blank correction. Once filled, the vials were sealed with rubber stoppers and aluminum caps, shaken and placed in an incubator (Shel Lab, Sheldon Manufacturing, Inc., Cornelius, OR, USA) at 39 ± 0.5 °C. The gas pressure in the headspace was measured manually by inserting a sterile needle connected to the pressure transducer at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 48, 60, 72, 96, and 144 h after inoculation. The volume of gas produced was estimated from the pressure values as proposed by López et al. [13].

To evaluate the fermentation kinetics, the exponential model proposed by France et al. [14] was adjusted to the gas production profiles:

$$G = A\left[1 - e^{-c(t-L)}\right] \quad (1)$$

where G is the cumulative gas production (ml g^{-1} DM) at time t (h), A is the asymptotic gas production (ml g^{-1} DM), c the fractional fermentation rate (per h), and L is the lag time (h).

The energy value (ME) and organic matter digestibility (OMD) were calculated from gas production after 24 h of incubation ($G24$) with complementary contents of crude protein and mineral matter, as suggested by Menke and Steingass [15]:

$$ME = 2.2 + 0.136 \times G24 + 0.057 \times CP + 0.029 \times CP^2 \quad (2)$$

$$OMD = 14.88 + 0.889 \times G24 + 0.45 \times CP + 0.0651 \times ash \quad (3)$$

where ME is metabolizable energy (MJ kg^{-1} DM), *G*24 the gas production after 24 h of incubation (ml 200 mg^{-1} DM), *CP* the crude protein content (% DM), *OMD* the organic matter digestibility (%), and *ash* the mineral matter content (% DM).

Following the same methods, another incubation of 16 h for the grains and of 24 h for other plant parts or for the whole-plant was performed. After incubation, the gas produced was measured with the aid of a sterile needle and a 100 mL syringe from which a sample of gas (10 mL) was collected from the headspace of the vial and then transferred to a Vacutainer® tube (BD Diagnostics, Franklin Lakes, NJ, USA). Immediately after opening the vials, a sample of the liquid contents (5 mL) from each vial was collected in tubes containing 100 µL of 20% sulfuric acid to stop the fermentation. The tubes were centrifuged at 4000× *g* for 10 min. A sample (1 mL) of the supernatant after centrifugation was collected into Eppendorf® microtubes (Eppendorf Company, Hamburg, Germany), and frozen at −20 °C for subsequent N-NH_3 analysis. Another sample (0.8 mL) was transferred to Eppendorf® microtubes adding 0.5 mL of an acidifying and deproteinizing solution (1% metaphosphoric acid and 0.2% crotonic acid in 0.5 M hydrochloric acid), and then frozen at −20 °C for volatile fatty acids (VFA) analysis.

2.1.4. Volatile Fatty Acids Analysis

Samples were thawed at 4 °C and centrifuged at 13,000× *g* for 15 min at 4 °C. The supernatant was transferred to 12 × 32 mm screw-cap vials with a capacity of 2 mL. The concentrations of valeric, isovaleric, butyric, isobutyric, propionic, and acetic acids were determined by gas chromatography on a Shimadzu GC 2010 (Shimadzu Corp., Kyoto, Japan) chromatograph equipped with an automatic injector, a flame ionization detector and a TR-FFAP semicapillary column (30 m × 0.53 mm × 1 µm; Supelco S.A., Barcelona, Catalonia, Spain).

2.1.5. Ammonia-Nitrogen (N-NH_3) Analysis

The concentration of N-NH_3 was determined by the colorimetry using the indophenol blue method [16]. The samples were thawed at 4 °C and centrifuged at 13,000× *g* for 15 min at 4 °C. An aliquot of 1 mL of the sample was mixed with 5 mL of a solution of phenol and sodium nitroprusside and with 4 mL of a solution of NaClO and NaOH. This mix was incubated in a water bath at 39 °C for 15 min. After this time, the absorbance was read on a spectrophotometer at a wavelength of 625 nm. To adjust the calculations, a calibration curve was generated with the data of a 50 mM ammonium sulfate solution.

2.1.6. Methane (CH_4) Analysis

The concentration of CH_4 in the fermentation gas was determined by gas chromatography using a Shimadzu GC-14B chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector and a Carboxen TM 1000 column (45/60, 2 m × 1/8 in., Sigma-Aldrich, Madrid, Spain). The temperatures were 170 °C, 200 °C and 200 °C in the column, injector and detector, respectively, and the flow of carrier gas (He) was 24 mL min^{-1}. Each gas sample (500 µL) was manually injected using Pressure-Lok® A-2 syringes (BGB Analytik, Rheinfelden, Germany), and the methane content was calculated by external calibration using a mixture of certified gases with 10% CH_4, 5% H_2, 25% N_2 and 60% CO_2 (Metal Carbos, S.A., Barcelona, Catalonia, Spain).

2.2. Experiment 2

2.2.1. Materials and Experimental Design

The same maize hybrids already described were used in random plots, with three replicates (plots) each. After harvesting plants in the dent grain stage (R5), they were chopped in a PN-Plus 2000 stationary forage instrument (NB Máquinas Ltd.a., Itapira, São Paulo, Brazil) and stored in polyvinyl chloride plastic mini-silos to provide a specific density of 600 kg of NM m^{-3}, which were sealed and stored for 180 days. After opening, a sample of 500 g was collected from each silo, which was weighed

and dried in a forced air oven at 55 °C until constant weight to determine the dry matter (DM) content. Afterwards, the samples were ground in a Willey mill with a mesh sieve of 1 mm.

2.2.2. Dry Matter and Fiber Degradability

The ruminal degradability of the DM and neutral detergent fiber (NDF) of the silages resulting from the harvesting of different maize hybrids at the dent grain stage was estimated by the in situ technique. Two adult bulls with permanent rumen cannulas were used and kept in individual stalls with access to water and maize silage ad libitum. Approximately 5 g of each sample, dried and ground to 1 mm, were weighed and placed in nylon bags measuring 12 × 8 cm and with 50 µm pores for subsequent rumen incubation [17]. The incubation times evaluated were 0, 2, 4, 6, 12, 24, 36, 48, 72, and 96 h.

After the samples were removed, they were washed in water with ice and brought to a forced air oven at 55 °C for 48 h. Then, the weight was recorded, and the DM disappearance was calculated. From the residue, the NDF was determined according to Van Soest et al. [11] assayed with a heat-stable amylase and expressed inclusive of residual ash. A first-order kinetics model was used for the estimation of ruminal degradation parameters from the DM and NDF disappearance rates at the different incubation times, using nonlinear regression by the Gauss-Newton method. The DM degradation parameters were estimated using the exponential equation proposed by Ørskov & McDonald [18]:

$$ISD_{DM} = a + b\left(1 - e^{-ct}\right) \quad (4)$$

where ISD_{DM} is the in situ DM disappearance (as % of DM incubated) at incubation time t (h), a is the intercept representing the fraction (%) that disappears instantly at $t = 0$, b is the potentially degradable fraction (%), and c is the fractional degradation rate (per h). As for NDF, there should not be an intercept, in this particular case, parameter a was excluded from the model [4].

The effective degradability (ED) of DM and NDF in the rumen was calculated as suggested by Ørskov & McDonald [18]:

$$ED_{DM} = a + b \times [c/(c + k)] \quad (5)$$

$$ED_{NDF} = b \times [c/(c + k)] \quad (6)$$

where k is the fractional passage rate of digesta through the rumen, assumed to be 2%, 5% or 8% per h for low, medium and high feed intake, respectively.

2.3. Statistical Analysis

The Milk2006 model [19,20] was used to estimate the net energy for lactation (NE_L) and the milk yield for each hybrid and harvest stage based on its chemical composition and digestibility. The Milk2006 model uses up-to-date information and has user-defined input flexibility for these estimations.

Data on DM and NDF degradability were analyzed using the nonlinear model (PROC NLIN) and regression (PROC REG) procedures of the SAS program (v. 9.2; SAS Institute Inc., Cary, NC, USA). For the analysis of variance, the following statistical model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \delta_k + \varepsilon_{ijk} \quad (7)$$

where Y_{ijk} is each individual observation for each variable related to hybrid i from maturity stage j in plot k; μ is the overall average; α_i is the effect of hybrid i (i = Maximus, Defender or Feroz); β_j is the effect of maturity stage j (j = R3, R4 or R5); γ_{ij} is the interaction of hybrid i with stage j; δ_k is the blocking effect of plot k (k = 1, 2, 3); and ε_{ijk} is the random error associated with each observation of Y_{ijk}. For those variables measured only at one maturity stage R5 (e.g., in situ degradability), the statistical model was simplified to $Y_{ik} = \mu + \alpha_i + \delta_k + \varepsilon_{ik}$. The procedure of general linear models (PROC GLM) of SAS was used for the analysis of variance (ANOVA), and the Tukey's test was used for the multiple comparison of means at 5% significance.

3. Results

3.1. In Vitro Digestibility of Plant Fractions

The in vitro digestibility of Feroz stems was not affected by maturity stage, while for the hybrids Maximus and Defender, the highest digestibility was observed when harvested at the earlier R3 stage (Table 1). At the R3 stage, the highest stem digestibility was observed for the Defender hybrid, while at the most advanced stages R4 and R5, stems of the Feroz hybrid were the most digestible ($p < 0.05$).

Table 1. In vitro dry matter digestibility (g kg^{-1} ± SD) of plant fractions with 48 h incubation.

Plant Part/Hybrid	Stage		
	R3	R4	R5
Stem			
Maximus	536 ± 10.9 ^{bA}	488 ± 10.1 ^{bC}	509 ± 7.2 ^{cB}
Defender	578 ± 7.0 ^{aA}	470 ± 12.0 ^{bB}	472 ± 9.8 ^{bB}
Feroz	494 ± 14.4 ^{c}	519 ± 10.2 ^{a}	545 ± 12.5 ^{a}
Leaves			
Maximus	754 ± 12.2 ^{A}	683 ± 29.7 ^{B}	645 ± 11.5 ^{B}
Defender	736 ± 8.7 ^{A}	672 ± 11.2 ^{B}	649 ± 5.7 ^{C}
Feroz	734 ± 13.0 ^{A}	714 ± 17.8 ^{A}	653 ± 08.0 ^{B}
Husk leaves			
Maximus	662 ± 6.7 ^{bA}	593 ± 18.4 ^{bB}	561 ± 27.3 ^{bB}
Defender	679 ± 8.5 ^{ab}	653 ± 29.7 ^{a}	639 ± 5.2 ^{a}
Feroz	696 ± 8.8 ^{aA}	559 ± 17.5 ^{bB}	545 ± 18.9 ^{bB}
Cob			
Maximus	674 ± 22.9 ^{aA}	609 ± 16.6 ^{A}	498 ± 31.8 ^{abB}
Defender	645 ± 13.8 ^{abA}	571 ± 2.1 ^{B}	520 ± 2.1 ^{aC}
Feroz	611 ± 18.7 ^{bA}	611 ± 28.4 ^{A}	443 ± 21.0 ^{bB}
Grain			
Maximus	972 ± 6.4 ^{A}	967 ± 8.5 ^{AB}	956 ± 6.4 ^{B}
Defender	970 ± 9.0	972 ± 5.4	946 ± 17.2
Feroz	970 ± 12.1	967 ± 4.8	956 ± 7.9
Whole-plant			
Maximus	677 ± 13.9	686 ± 6.7	734 ± 20.1
Defender	655 ± 13.7	676 ± 13.2	706 ± 10.3
Feroz	664 ± 6.2	712 ± 5.6	742 ± 1.7

$^{a, b, c,}$ Within the same column, for each plant fraction, means not sharing common superscript letters are significantly different ($p < 0.05$); $^{A, B, C,}$ Within the same row means not sharing common superscript letters are significantly different ($p < 0.05$).

There were no significant differences among hybrids in leaf digestibility, and in all cases the highest digestibility was observed at the R3 stage, although for Feroz hybrid, the difference in the leaf digestibility between stages R3 and R4 was not significant. There was a decline in leaf digestibility with maturity, the decrease from R4 to R5 was significant for the Defender hybrid ($p < 0.05$), while the values for the Maximus hybrid remained similar. The Defender hybrid did not exhibit changes in the husk digestibility with increasing maturity and showed higher values than the other hybrids at stages R4 and R5. Maximus and Feroz hybrids had higher husk digestibility at the earliest stage, with no difference between the R4 and R5 stages. The cob digestibility values were lower at the later stage for all hybrids, and for the Feroz than the other hybrids. Grain and whole-plant digestibility were not significantly different among maize hybrids at any stage. The Maximus hybrid showed decreasing grain digestibility as maturity stage was more advanced.

3.2. In Vitro Digestibility of Grain and Whole-Plant

The grain digestibility analyzed by the method of Goering & Van Soest [8] showed no difference among hybrids (Table 2). However, grain digestibility decreased with increasing maturity, except for the Feroz hybrid, which did not differ between stages R4 and R5. When analyzed by the method proposed by Tilley & Terry [9], differences among maturity stages were not significant for the Maximus and Feroz hybrids, whereas digestibility of grain of Defender hybrid was higher in the R4 stage than at stages R3 and R5. At stages R3 and R5, there were no differences in grain digestibility among the evaluated hybrids.

Table 2. In vitro digestibility (g kg^{-1} ± SD) of dry matter of the grain (16 h) and the whole-plant (24 h) by the methods of Goering & Van Soest [8] and Tilley & Terry [9] modified.

	Grain			Whole-Plant		
Hybrid	**R3**	**R4**	**R5**	**R3**	**R4**	**R5**
Goering & Van Soest [8]						
Maximus	955 ± 3.1 ^{A}	944 ± 3.5 ^{AB}	922 ± 12.2 ^{B}	588 ± 7.8 ^{B}	580 ± 15.6 ^{bB}	693 ± 7.1 ^{aA}
Defender	961 ± 6.4 ^{A}	946 ± 8.8 ^{AB}	927± 7.6 ^{B}	576 ± 15.3 ^{B}	600 ± 16.1 ^{bAB}	635 ± 22.1 ^{bA}
Feroz	962 ± 5.2 ^{A}	940 ± 11.1 ^{B}	935 ± 0.8 ^{B}	560 ± 1.3 ^{B}	637 ± 30.0 ^{aAB}	685 ± 3.0 ^{aA}
Tilley & Terry [9] **modified**						
Maximus	930 ± 2.9	901 ± 13.9 ^{b}	906 ± 20.5	643 ± 9.3 ^{aB}	646 ± 12.2 ^{bB}	732 ± 16.9 ^{aA}
Defender	926 ± 12.5 ^{AB}	943 ± 4.4 ^{aA}	900 ± 15.5 ^{B}	633 ± 12.5 ^{aB}	625 ± 4.4 ^{bB}	642 ± 15.5 ^{cA}
Feroz	928 ± 6.4	918 ± 16.4 ^{ab}	914 ± 12.8	603 ± 7.0 ^{bB}	714 ± 19.9 ^{aA}	717 ± 13.8 ^{bA}

$^{a, b, c,}$ Within the same column, for each in vitro technique, means not sharing common superscript letters are significantly different ($p < 0.05$); $^{A, B, C,}$ Within the same row, for either grain or whole-plant, means not sharing common superscript letters are significantly different ($p < 0.05$).

The whole-plant digestibility increased with increasing maturity for the three evaluated hybrids. At the R5 stage, the Maximus hybrid showed the highest whole-plant digestibility, whereas at R4 the highest digestibility values were observed for the whole-plant of Feroz hybrid. In contrast, the Feroz whole-plant was the least digestible at R3 according to the method of Tilley & Terry [9].

3.3. Gas Production Kinetics

The asymptotic gas production of the Maximus whole-plant did not differ among maturity stages (Table 3), whereas for the other hybrids, the highest ($p < 0.05$) gas production was observed in stage R3, with no difference between stages R4 and R5. The asymptotic gas production was highest for the Feroz hybrid at R3, and for the Maximus hybrid at the other maturity stages. For grain, the asymptotic gas production was highest for the Maximus hybrid at all the stages, whereas the Feroz hybrid showed the lowest values.

The lowest gas production was observed in the latest maturity stage (R5). There was a decrease with maturity in the fermentation rate of the grain of the Defender maize. For the other hybrids, the differences among stages were not significant. Fermentation rates were slowest with Feroz corn grain for all stages, not differing from that of Defender at R5. In relation to the whole-plant, the Defender and Feroz hybrids showed the fastest fermentation rates at R3 and the slowest at the R5 stage. Except for the Defender hybrid, lag time tended to increase with maturity. When comparing hybrids, Feroz showed in most cases the shortest lag time. The gas production at 24 h of grain incubation decreased with increasing maturity stage for the Maximus and Defender hybrids. Among hybrids, the highest values of *G*24 were observed for Maximus grain and whole-plant. The differences among maturity stages in *G*24 of the whole-plant were variable for each maize hybrid.

The ME content of maize whole-plant was decreased in R5 compared with in R3 and R4 for the three hybrids. Regardless of the maturity stage, the Maximus hybrid was always superior to the other hybrids in terms of its energy value. A similar behavior was observed for the OMD, where the Maximus hybrid showed the highest estimated digestibility.

Table 3. In vitro fermentation kinetics (estimated from gas production curves), and estimated metabolizable energy (ME) and organic matter digestibility (OMD).

Hybrid	Grain			Whole-Plant		
Maturity	**R3**	**R4**	**R5**	**R3**	**R4**	**R5**
***A*, ml g^{-1} DM incubated**						
Maximus	433 ± 3.4 ^{aA}	421 ± 5.4 ^{aB}	413 ± 2.9 ^{aB}	359 ± 1.0 ^{b}	363 ± 5.6 ^{a}	363 ± 4.3 ^{a}
Defender	404 ± 6.3 ^{bA}	412 ± 3.2 ^{aA}	380 ± 4.1 ^{bB}	365 ± 5.5 ^{bA}	312 ± 1.8 ^{bB}	322 ± 6.6 ^{bB}
Feroz	369 ± 2.7 ^{c}	366 ± 2.8 ^{b}	365 ± 2.3 ^{c}	407 ± 4.5 ^{aA}	309 ± 1.8 ^{bB}	318 ± 2.4 ^{bB}
***c*, h^{-1}**						
Maximus	0.057 ± 0.0004 ^{b}	0.056 ± 0.0013 ^{a}	0.056 ± 0.0008 ^{a}	0.037 ± 0.0006 ^{aC}	0.040 ± 0.0001 ^{aB}	0.043 ± 0.0003 ^{aA}
Defender	0.062 ± 0.0006 ^{aA}	0.057 ± 0.0009 ^{aB}	0.043 ± 0.0008 ^{bC}	0.037 ± 0.0002 ^{aA}	0.025 ± 0.0003 ^{bC}	0.031 ± 0.0005 ^{bB}
Feroz	0.047 ± 0.0003 ^{c}	0.047 ± 0.0024 ^{b}	0.046 ± 0.0019 ^{b}	0.035 ± 0.0009 ^{bA}	0.024 ± 0.0014 ^{bC}	0.029 ± 0.0008 ^{bB}
***L*, h**						
Maximus	4.92 ± 0.11 ^{aC}	5.66 ± 0.29 ^{abB}	6.29 ±0.10 ^{aA}	2.74 ± 0.16 ^{aB}	2.32 ± 0.18 ^{aB}	3.60 ± 0.35 ^{aA}
Defender	5.25 ± 0.29 ^{aAB}	6.05 ± 0.29 ^{aA}	4.54 ± 0.32 ^{bB}	2.75 ± 0.18 ^{aA}	0.70 ± 0.11 ^{cC}	1.38 ± 0.23 ^{bB}
Feroz	3.26 ± 0.17 ^{bB}	4.60 ± 0.50 ^{bA}	4.57 ± 0.17 ^{bA}	1.18 ± 0.12 ^{bB}	1.44 ± 0.10 ^{bA}	1.17 ± 0.18 ^{bB}
***G*24, ml g^{-1} DM incubated**						
Maximus	285 ± 2.6 ^{aA}	270 ± 3.8 ^{aB}	259 ± 3.8 ^{aC}	188 ± 2.0 ^{aC}	196 ± 3.3 ^{aB}	207 ± 1.9 ^{aA}
Defender	274 ± 3.9 ^{bA}	260 ± 2.8 ^{aB}	201 ± 3.5 ^{cC}	184 ± 3.2 ^{aA}	135 ± 1.3 ^{bC}	162 ± 3.2 ^{bB}
Feroz	223 ± 1.2 ^{c}	217 ± 5.7 ^{b}	223 ± 5.4 ^{b}	220 ± 1.8 ^{bB}	134 ± 5.4 ^{aA}	155 ± 1.7 ^{bB}
EM, MJ kg^{-1} DM						
Maximus	-	-	-	11.37 ± 0.06 ^{aA}	10.05 ± 0.09 ^{aB}	9.66 ± 0.05 ^{aC}
Defender	-	-	-	9.49 ±0.06 ^{bA}	8.46 ± 0.03 ^{bC}	8.77 ± 0.09 ^{bB}
Feroz	-	-	-	9.57 ± 0.05 ^{bAB}	9.96 ± 0.47 ^{aA}	8.97 ± 0.15 ^{bB}
OMD, %						
Maximus	-	-	-	53.6 ± 0.37 ^{aB}	53.9 ± 0.57 ^{aAB}	55.1 ± 0.31 ^{aA}
Defender	-	-	-	51.6 ± 0.40 ^{bA}	43.1 ± 0.23 ^{bC}	47.4 ± 0.57 ^{bB}
Feroz	-	-	-	43.8 ± 0.35 ^{cB}	57.3 ± 3.06 ^{aA}	46.5 ± 0.96 ^{bB}

A: asymptotic gas production; *c*: fractional fermentation rate; *L*: lag time; *G*24: gas production with 24 h of incubation. $^{a, b, c,}$ Within the same column, for each parameter, means not sharing common superscript letters are significantly different ($p < 0.05$); $^{A, B, C,}$ Within the same row, for either grain or whole-plant, means not sharing common superscript letters are significantly different ($p < 0.05$).

3.4. Fermentation End-Products

There were few significant differences among hybrids in fermentation end-products output (Table 4). For grain, gas production was lowest for Defender and total VFA highest for Feroz at R5. For the whole-plant, Feroz showed higher total gas and methane production than the other hybrids at R4, but at R5 the values for Feroz were the lowest.

Table 4. Production of total VFA, methane and ammonia after 16 h of grain incubation and 24 h of whole-plant incubation.

	Grain			Whole-Plant		
Hybrid	**R3**	**R4**	**R5**	**R3**	**R4**	**R5**
Total fermentation gas, mmol g^{-1} DM incubated						
Maximus	5.58 ± 0.32 ^{A}	4.86 ± 0.45 ^{B}	5.01 ± 0.41 ^{aB}	4.16 ± 0.26 ^{C}	4.59 ± 0.06 ^{bB}	5.78 ± 0.17 ^{aA}
Defender	5.41 ± 0.48 ^{A}	5.00 ± 0.56 ^{AB}	4.53 ± 0.41 ^{bB}	3.91 ± 0.14 ^{C}	4.60 ± 0.14 ^{bB}	4.95 ± 0.26 ^{aA}
Feroz	5.69 ± 0.41 ^{A}	5.14 ± 0.32 ^{B}	5.21 ± 0.42 ^{aB}	4.37 ± 0.10 ^{B}	5.87 ± 0.22 ^{aA}	2.64 ± 0.40 ^{bC}
Methane, µmol g^{-1} DM incubated						
Maximus	595 ± 92.6 ^{A}	437 ± 81.3 ^{B}	452 ± 67.3 ^{B}	494 ± 56.3 ^{B}	575 ± 38.5 ^{bB}	767 ± 60.5 ^{aA}
Defender	582 ± 27.7 ^{A}	436 ± 63.2 ^{B}	432 ± 79.8 ^{B}	460 ± 50.0 ^{B}	635 ± 20.3 ^{bA}	616 ± 82.5 ^{aA}
Feroz	616 ± 69.9 ^{A}	455 ± 40.5 ^{B}	436 ± 43.3 ^{B}	584 ± 29.5 ^{B}	796 ± 72.5 ^{aA}	324 ± 62.2 ^{bC}
Methane, mmol mol^{-1} gas						
Maximus	106.2 ± 10.4	89.5 ± 8.4	90.4 ± 9.3	119 ± 6.7	125 ± 9.4	134 ± 6.7
Defender	108.5 ± 4.0 ^{A}	87.1 ± 5.1 ^{B}	95.3 ± 9.3 ^{AB}	118 ± 10.2	139 ± 2.2	125 ± 13.2
Feroz	108.1 ± 9.2 ^{A}	89.1 ± 7.2 ^{B}	83.5 ± 2.9 ^{B}	134 ± 3.8	131 ± 2.7	126 ± 11.7
Total VFA, mmol g^{-1} DM incubated						
Maximus	5.39 ± 0.11	5.28 ± 0.57	5.60 ± 0.77 ^{b}	5.16 ± 0.63	5.68 ± 0.76	6.22 ± 0.24
Defender	5.99 ± 0.86 ^{A}	5.48 ± 0.80 ^{B}	5.49 ± 0.68 ^{bB}	5.09 ± 0.42 ^{B}	5.41 ± 0.16 ^{AB}	5.70 ± 0.36 ^{A}
Feroz	5.89 ± 0.45	5.90 ± 0.56	6.08 ± 0.82 ^{a}	5.37 ± 0.32 ^{B}	6.78 ± 0.48 ^{A}	5.91 ± 0.66 ^{AB}
Methane, mmol mol^{-1} VFA						
Maximus	110.5 ± 18.8 ^{A}	83.2 ± 8.4 ^{B}	81.6 ± 1.9 ^{B}	98.0 ± 23.5	103 ± 10.1	125 ± 7.9 ^{a}
Defender	99.0 ± 12.2	79.6 ± 0.4	79.3 ± 6.2	91.9 ± 14.6 ^{B}	119 ± 6.7 ^{A}	111 ± 13.7 ^{aAB}
Feroz	104.8 ± 11.6 ^{A}	78.2 ± 5.9 ^{B}	72.0 ± 3.2 ^{B}	109 ± 2.7 ^{A}	118 ± 5.4 ^{A}	56.4 ± 16.4 ^{bB}
N-NH_3, mg N L^{-1}						
Maximus	106 ± 11.6	115 ± 3.4	128 ± 5.6	208 ± 8.2 ^{A}	197 ± 3.4 ^{A}	157 ± 6.1 ^{B}
Defender	116 ± 4.8 ^{B}	126 ± 8.7 ^{AB}	144 ± 8.3 ^{A}	203 ± 7.7 ^{A}	183 ± 10.9 ^{AB}	165 ± 10.6 ^{B}
Feroz	106 ± 2.2	130 ± 7.3	131 ± 14.8	201 ± 6.9 ^{A}	197 ± 9.1 ^{A}	154 ± 5.9 ^{B}

$^{a, b, c,}$ Within the same column, for each parameter, means not sharing common superscript letters are significantly different ($p < 0.05$); $^{A, B, C,}$ Within the same row, for either grain or whole-plant, means not sharing common superscript letters are significantly different ($p < 0.05$).

The general trend was a decrease in gas, methane and VFA production with maturity when grain was fermented, and a slight increase when the whole-plant was digested. However, this trend was variable for each maize hybrid (Table 4). Methane concentration in fermentation gas tended to decrease with maturity for corn grain, with small variations among maturity stages for whole-plant or among hybrids. When expressed per mmol of VFA produced, methane decreased with maturity when Maximus and Feroz grain was fermented, whereas for the whole-plant, the differences among maturity stages were variable for each hybrid (Table 4). The N-NH_3 concentration increased with increasing maturity with Defender corn grain. An increase in N-NH_3 was observed for whole-plant incubation for all hybrids, so that the highest values were observed in R3.

The molar proportion of acetate was not affected by grain maturity for Maximus and Defender hybrids and tended to decrease with maturity with the Feroz. In most cases, when corn grain was incubated, the molar proportions of propionate and valerate increased with maturity, whereas that of butyrate was decreased (Table 5). When the whole-plant of Maximus and Defender hybrids were incubated, molar proportions of propionate and butyrate were increased and that of acetate was decreased with maturity. In the case of Feroz whole-plant, the highest acetate and the lowest propionate and butyrate were observed at R5. As a result, the acetate to propionate ratio was decreased with maturity in all hybrids, with significant differences in most cases. The exception was the whole-plant of Feroz hybrid, for which the highest acetate to propionate ratio was observed at R5.

Table 5. Molar proportions of volatile fatty acids (mmol per mol of total VFA) after 16 h of grain incubation or 24 h of whole-plant incubation.

	Grain			Whole-Plant		
	R3	**R4**	**R5**	**R3**	**R4**	**R5**
Acetate						
Maximus	575 ± 0.8	574 ± 1.5	565 ± 15.1	629 ± 5.9 ^{A}	615 ± 5.9 ^{aA}	584 ± 5.3 ^{bB}
Defender	565 ± 16.3	574 ± 4.3	574 ± 7.4	627 ± 11.5 ^{A}	607 ± 3.6 ^{aB}	595 ± 8.6 ^{bC}
Feroz	577 ± 2.8 ^{A}	571 ± 7.1 ^{AB}	562 ± 9.1 ^{B}	621 ± 2.1 ^{B}	580 ± 1.4 ^{bB}	641 ± 7.9 ^{aA}
Propionate						
Maximus	212 ± 5.1 ^{B}	238 ± 9.4 ^{AB}	245 ± 9.7 ^{bA}	207 ± 7.7 ^{B}	216 ± 11.1 ^{AB}	231 ± 2.7 ^{aA}
Defender	231 ± 11.8 ^{B}	239 ± 10.1 ^{AB}	248 ± 14.1 ^{abA}	207 ± 12.9 ^{B}	217 ± 5.4 ^{AB}	228 ± 10.3 ^{aA}
Feroz	215 ± 4.8 ^{B}	248 ± 13.0 ^{A}	262 ± 10.4 ^{aA}	200 ± 9.2 ^{B}	239 ± 18.6 ^{A}	195 ± 1.0 ^{bB}
Butyrate						
Maximus	179 ± 2.9 ^{aA}	156 ± 4.1 ^{B}	153 ± 3.7 ^{aB}	126 ± 9.3 ^{B}	131 ± 0.4 ^{bB}	156 ± 3.1 ^{aA}
Defender	171 ± 4.2 ^{bA}	154 ± 3.3 ^{B}	139 ± 2.3 ^{bC}	126 ± 4.7 ^{C}	137 ± 3.4 ^{abAB}	142 ± 5.8 ^{bA}
Feroz	175 ± 2.4 ^{abA}	154 ± 5.8 ^{B}	151 ± 3.0 ^{aB}	139 ± 3.4 ^{B}	149 ± 9.3 ^{aA}	122 ± 6.6 ^{cC}
Valerate						
Maximus	9.6 ± 0.02 ^{bB}	10.1 ± 0.19 ^{bB}	11.1 ± 0.38 ^{bA}	10.4 ± 0.46	10.7 ± 0.17	10.6 ± 0.12
Defender	10.8 ± 0.64 ^{a}	10.6 ± 0.33 ^{a}	12.0 ± 0.06 ^{a}	11.0 ± 0.52	10.8 ± 0.30	10.9 ± 0.19
Feroz	9.9 ± 0.07 ^{abC}	10.8 ± 0.27 ^{aB}	11.7 ± 0.04 ^{aA}	11.1 ± 0.77	11.5 ± 0.68	11.3 ± 0.90
Iso-acids						
Maximus	24.8 ± 2.3	22.8 ± 6.1	26.1 ± 3.1	26.3 ± 4.4 ^{AB}	27.5 ± 4.8 ^{A}	18.9 ± 1.6 ^{B}
Defender	22.5 ± 5.8	22.3 ± 3.0	26.3 ± 7.2	28.4 ± 5.1	28.8 ± 5.0	24.2 ± 7.8
Feroz	22.8 ± 3.5	16.6 ± 8.1	13.3 ± 4.1	29.2 ± 4.3 ^{AB}	20.7 ± 7.9 ^{B}	31.2 ± 1.4 ^{A}
Acetate to propionate ratio						
Maximus	2.71 ± 0.06 ^{A}	2.41 ± 0.09 ^{AB}	2.32 ± 0.15 ^{B}	3.04 ± 0.09 ^{A}	2.86 ± 0.17 ^{A}	2.53 ± 0.05 ^{bB}
Defender	2.45 ± 0.19	2.41 ± 0.12	2.32 ± 0.16	3.04 ± 0.24 ^{A}	2.79 ± 0.08 ^{AB}	2.62 ± 0.16 ^{bB}
Feroz	2.69 ± 0.07 ^{A}	2.31 ± 0.14 ^{B}	2.15 ± 0.12 ^{B}	3.11 ± 0.15 ^{A}	2.57 ± 0.17 ^{B}	3.30 ± 0.03 ^{aA}

$^{a, b, c,}$ Within the same column, for each parameter, means not sharing common superscript letters are significantly different ($p < 0.05$); $^{A, B, C,}$ Within the same row, for either grain or whole-plant, means not sharing common superscript letters are significantly different ($p < 0.05$).

3.5. Energy and Estimated Milk Yield

The estimated net energy differed among maize hybrids at the R3 and R5 stages (Table 6). At R3, the Feroz hybrid showed the highest NE content, while at R5, it was the lowest. For all hybrids, the net energy increased with increasing maturity, especially the Maximus hybrid at the R5 stage. The estimated milk yield, either per ton of DM or per hectare, also increased with increasing maturity ($p < 0.05$), with no significant differences among the hybrids for milk yield in kg ha^{-1}.

Table 6. Values of net energy ($NE_{Ll\text{-}3}$) and expectation of milk yield estimated by the Milk 2006 model.

Hybrid	**Stage**		
	R3	**R4**	**R5**
$NE_{Ll\text{-}3\times}$, Mcal kg^{-1} maize			
Maximus	1.196 ± 0.004 ^{bC}	1.318 ± 0.006 ^{B}	1.494 ± 0.010 ^{aA}
Defender	1.162 ± 0.004 ^{cC}	1.302 ± 0.008 ^{B}	1.447 ± 0.013 ^{abA}
Feroz	1.237 ± 0.004 ^{aC}	1.326 ± 0.013 ^{B}	1.437 ± 0.021 ^{bA}
Milk yield, g milk kg^{-1} DM maize			
Maximus	1029 ± 5.3 ^{bC}	1191 ± 8.6 ^{B}	1425 ± 13.8 ^{aA}
Defender	984 ± 5.4 ^{cC}	1170 ± 10.7 ^{B}	1362 ± 17.7 ^{abA}
Feroz	1083 ± 5.0 ^{aC}	1202 ± 17.5 ^{B}	1349 ± 28.1 ^{bA}
Milk yield, kg ha^{-1}			
Maximus	17,741 ± 1148 ^{C}	28,941 ± 2100 ^{B}	44,038 ± 5334 ^{A}
Defender	14,445 ± 207 ^{C}	27,020 ± 659 ^{B}	41,090 ± 4428 ^{A}
Feroz	15,488 ± 2322 ^{C}	28,109 ± 2860 ^{B}	40,103 ± 2381 ^{A}

$^{a, b, c,}$ Within the same column, for each parameter, means not sharing common superscript letters are significantly different ($p < 0.05$); $^{A, B, C,}$ Within the same row means not sharing common superscript letters are significantly different ($p < 0.05$).

For the yield of milk per ton of DM harvested, the Feroz hybrid showed the highest value at R3, while at R5 the highest value was for the Maximus hybrid. From stages R3 to R5, an increase of 38% in estimated milk yield per ton of DM was observed for the Maximus and Defender hybrids and of 24% for the Feroz hybrid.

3.6. In Situ Ruminal Degradation of Silage

The silage resulting from harvesting maize whole-plant at the dent grain stage (R5) was different among the hybrids in terms of chemical composition, with a relatively low NDF content and high starch concentration for the Maximus hybrid [21].

The ruminal degradation kinetics data are shown in Table 7. Fraction "*a*" was higher for the Maximus hybrid than for the Feroz hybrid, and fraction "*b*" was higher for the Feroz hybrid than for the Defender hybrid. As the passage rate was faster (5% and 8%, respectively), ED was higher for the Maximus and Defender than for the Feroz maize silage. The potential degradability of NDF was higher for the Maximus than for the Defender hybrid. Degradation rate of fraction "*b*" or the ED were not affected by the hybrid.

Table 7. In situ ruminal degradation kinetics of dry matter (DM) and neutral detergent fiber (NDF) of silage of different maize hybrids harvested at dent grain stage.

		Silage Hybrid				
	Parameter	**Maximus**	**Defender**	**Feroz**	**S.E.M**	***p*-Value**
DM	***a*, %**	28.11 ^{a}	26.05 ^{ab}	24.78 ^{b}	0.792	0.050
	***b*, %**	54.68 ^{ab}	50.66 ^{b}	62.69 ^{a}	2.888	0.048
	***c*, h^{-1}**	0.018	0.019	0.015	0.001	0.582
	U, %	17.21 ^{ab}	23.29 ^{a}	12.83 ^{b}	2.544	0.006
	ED, $k = 0.02$ h^{-1}	52.39	50.42	49.23	0.752	0.078
	ED, $k = 0.05$ h^{-1}	41.68 ^{a}	39.81 ^{ab}	37.46 ^{b}	0.996	0.009
	ED, $k = 0.08$ h^{-1}	37.56 ^{a}	35.63 ^{a}	33.38 ^{b}	0.987	0.003
NDF	***b*, %**	53.63 ^{a}	44.20 ^{b}	49.20 ^{ab}	1.222	0.019
	***c*, h^{-1}**	0.125	0.178	0.139	0.013	0.259
	U, %	46.37 ^{b}	55.80 ^{a}	50.80 ^{ab}	1.222	0.018
	ED, $k = 0.02$ h^{-1}	42.21	42.42	41.68	0.179	0.101

a: soluble fraction; *b*: potentially degradable fraction; *c*: fractional degradation rate; U: undegradable fraction; ED: effective degradability; *k*: fractional passage rate. $^{a, b,}$ Within the same row, for either DM or NDF, means not sharing common superscripts are significantly different ($p < 0.05$).

4. Discussion

4.1. In Vitro Digestibility

The Maximus hybrid is commercially known for having a more pronounced stay-green compared to that of the other hybrids evaluated herein. Thus, it could be expected that the vegetative fraction of this hybrid could be more digestible than that of the other hybrids. Although this difference in favor of the Maximus hybrid was not always observed when IVDMD was determined; other results such as fermentation kinetics or the production of gas or VFA by fermentation, seem to confirm the superiority of the Maximus over the other two hybrids evaluated. In a review, Khan et al. [1] suggested that a correlation between the stay-green and digestibility of vegetative fractions of plants was not always observed, but stressed the importance of the maturity stage and the DM obtained.

In general, the decrease in the digestibility of the vegetative fractions with increasing maturity was related to the accumulation of cell wall components in leaves and stems and to the accrual of nonstructural carbohydrates to the grains [3]. Therefore, the general relationship between the optimal stage of maturity and the contribution of each fraction to the whole-plant and its particular digestibility

must be observed. As an example, Zeoula et al. [22] concluded that hybrids with highly digestible stems can reduce concentrate use by up to 1.5 kg per animal day^{-1}.

The decrease observed in grain digestibility in the Maximus hybrid was also expected in the other hybrids because increasing maturity increases the grain hardness (vitreousness) due to protein matrix accumulation around the endosperm, making it difficult for microorganisms to attack starch granules. [23]. Due to their increasing proportion in the plant, the grains have a critical contribution to the quality of the whole-plant and therefore, even with observed differences between hybrids and maturity stages for all fractions except for that of grains, the digestibility of the whole-plant was unchanged. However, it should be noted that the digestibility of the whole-plant is primarily dependent on the quality of each fraction and secondarily on their proportions [3].

Contrary to what was observed after 48 h of incubation, when the in vitro digestibility was determined at shorter incubation times in rumen fluid (16 h for the grain and 24 h of the whole-plant, according to their expected residence times in the rumen), the differences between hybrids and maturity stages were more remarkable. It is worth mentioning that the methodology of Goering & Van Soest [8] provided grain digestibility values numerically higher than those found by the Tilley & Terry [9] technique, while the opposite was observed for whole-plants. Holden [10] highlighted a 5.3% difference in grain digestibility between these methodologies, with that of Tilley & Terry being superior to that of Goering & Van Soest [8], but no significant difference was observed. Based on these data, Holden [10] proposed changes in the first technique, which have been currently adopted. Through the technique proposed by Goering & Van Soest [8], regardless of hybrid, grain digestibility decreased with increasing grain maturity. With the method proposed by Tilley & Terry [9], the grain of the Defender hybrid exhibited the highest digestibility at R4, which can be explained by being the stage with fastest grain starch accumulation, in comparison with R3 and R5 stages. The digestibility of the whole-plant after 24 h of incubation increased with increasing maturity and seemed to be related to the proportion of grains in the plant. The data presented by Peyrat et al. [24] demonstrated that starch digestibility does not exhibit statistical differences between maturity stages although the concentration of starch in the plant changes significantly with growth. Di Marco et al. [4] reported similar results up to the stage preceding the kernel black layer formation, when the highest concentration of starch but low-quality fiber were observed in the plant. Our data corroborate the review by Khan et al. [1], showing that the advance of the harvest stage provides better DM digestibility, suggesting silage production that can potentially support a higher milk yield by the dairy cow. It should be noted that the digestibility of the whole-plant is determined by both the quality of the vegetative fraction and the increasing proportion of the most digestible fraction (i.e., the grain), as demonstrated in a number of studies [1,2,23].

In situ degradation kinetics of silage from the three hybrids were assessed at stage R5, because at this stage silages were produced with suitable DM intake and acceptable contents of starch and digestible fiber [21]. Krämer-Schmid et al. [25] highlighted the importance of the potential degradation of NDF and of the starch concentration on DM degradability to explain the differences found among various maize hybrids. The process of silage fermentation promotes partial hydrolysis of the matrix proteins in the endosperm [26], favoring faster starch degradation. Thus, hybrids with more starch in the whole-plant at harvest provide silages with a greater soluble or instantly degradable DM, and would explain the increased DM degradability at fast passage rates of hybrids (e.g., Maximus), showing a higher "a" fraction. However, differences were not observed in the degradability of DM at a slow passage rate, where the potential fiber degradation would be more influential.

4.2. Ruminal Fermentation

Fermentation kinetics data, when performed by the in vitro gas production technique, are very valuable, in addition to allowing for the evaluation and screening of a large number of samples with low cost and high repeatability. These data usually show that the evaluation of hybrids and maturity stages is well correlated with the chemical composition [5]. With a more advanced plant maturity, there is an increase in the DM content and the proportion of grain in the harvested biomass. Corn grain

vitreousness is increased with maturity and, consequently, starch utilization may be reduced, limiting the amount of substrate available for fermentation and gas production [27]. However, the proportion of grain in total DM is also increased, with a reduction of the proportion of more fibrous parts in the whole-plant. Grain provides more a fermentable substrate for gas production when compared to the vegetative fraction, explaining the inverse pattern observed between the production of gas from grains or from the maize whole-plant with increasing maturity. Although the classification of grain maturity stage of maize based on its hardness is important, differences may exist even within a particular stage. According to Pôssas et al. [27], each hybrid advances in protein matrix deposition at a different rate, justifying the differences in the fermentation kinetics observed among the evaluated hybrids. For example, with the Feroz hybrid, the asymptotic gas production did not differ among stages, whereas with the Maximus and Defender hybrids, the highest and lowest values of this parameter were observed at R3 and R5, respectively. Likewise, *G*24 with grain decreased with maturity for the Maximus and Defender hybrids, but there were no difference among stages with the Feroz hybrid. Assuming that the organic matter fermented in the rumen is mostly composed of carbohydrates as proposed by Wolin [28], it can be expected that ensiling material with more grain, and thus most likely with more starchy carbohydrates, would provide more fermentable matter in the rumen, thus resulting in more gas production. The whole-plant of the Maximus hybrid had a greater proportion of grain (unpublished data) and is characterized by a more pronounced stay-green than the other two hybrids. Therefore, it could be expected that the whole-plant of this hybrid would be degraded in the rumen to a greater extent, and more *G*24 volumes were recorded with Maximus than with the other hybrids at the three maturity stages. The gas production technique has been more sensitive than gravimetric in vitro digestibility techniques to discriminate the maize hybrids according to the extent of their degradation in the rumen.

The observed CH_4 production when grain is fermented showed that with maturity, starch degradability may be reduced, thereby reducing CH_4 output at later stages of maturity, although no differences among hybrids in CH_4 production were observed. Increasing starch content in ruminant diets is mentioned by some authors [29] as an effective way to decrease CH_4 emission per unit of fermented matter. In our study, methane was increased at later stages of maturity in Maximus and Defender hybrids, most likely due to the increased grain proportion to the biomass, so that more organic matter is readily fermentable regardless of the likely reduction in fiber NDF degradation [30,31]. Hatew et al. [31] suggested that advancing maize plant maturity decreases CH_4 emissions in dairy cows mainly by reducing the ruminal fractional rate of starch degradation, causing an increased escape of starch to the duodenum. However, in in vitro batch cultures, starch cannot escape fermentation and is fully available for microbial digestion; thus, methane production is cumulative [30]. Differences between in vitro and ruminal data are continuously described [7] but do not invalidate the method to investigate the differences between feeds.

Advancing plant maturity tends to reduce NDF content and increase starch content, determining the VFA production profile, namely, increasing propionate and decreasing acetate production [31]. The increase in grain concentration in the plant induces a reduction in rumen liquid pH and generally favors propionate production at the expense of acetate [7,32]. Structural carbohydrates are the main source for acetate and butyrate production from rumen fermentation, and their synthesis results in an increase in H_2 production, which is used together with CO_2 by methanogenic microorganisms for CH_4 production [7]. An increased fiber content in the plant (often caused by the advancement of the maturity stage) not only increases CH_4 production but also increases the molar proportions of butyrate and acetate. The total VFA production was different between maturity stages for the evaluated hybrids, showing that comparisons of harvest stages with only one hybrid utilized for silage production should be interpreted with caution. Valerate and iso-acids originated from protein fermentation, with the amino acid proline (present in large quantities in prolamins) as one of the main sources [33]. Changes in protein degradation are related to differences in ammonia concentration

in batch cultures. However, it was not possible to observe noticeable differences among hybrids or maturity stages in these parameters.

4.3. Energy and Estimated Milk Yield

One of the main differences among hybrids when estimating their energy value is starch [34]. The starch content of the whole-plant was analyzed with an enzymatic kit, and data were entered into the Milk2006 model [19,20]. The different hybrids showed more or less energy value at different maturity stages, which also affected the estimation of milk yield per ton of DM harvested. Our data would suggest a higher grain yield for the Feroz than for the other hybrids at stage R3, but a higher grain yield for the Maximus hybrid at stage R5, and both exhibited higher energy values at the corresponding stages. Slight variations in fiber digestibility may not be sufficient to influence the estimation of milk yield potential [35], whereas the gradual increase in the proportion of grains in the plant with increasing maturity showed its importance. As the grain is the most energetic fraction, the most advanced stages showed the highest values for both net energy content and potential milk yield, even with a tendency for fiber utilization to be decreased. According to Johnson et al. [36], the highest nutritional value of most maize hybrids is attained when plants have between 33 and 36% DM. This occurred at the R4 and R5 stages for the hybrids evaluated in this study. Finally, the yield of each hybrid should be taken into account for decision making, as the differences found in DM yield per area were balanced by the differences observed between hybrids when estimating the milk yield per hectare.

5. Conclusions

Under favorable ensiling conditions, even if the vegetative fractions differ among hybrids and maturity stages, the differences brought about by the grain yield potential seem to be more influential on the quality of the silage. The energy content, in vitro digestibility and fermentation kinetics indicate that the Maximus VIP3 hybrid is the most suitable for silage production when harvested at the dent grain stage. Regardless of the hybrid, harvesting the maize at the dent grain stage results in enhanced digestibility of the whole-plant, due to the higher proportion of grain in the ensiling material. At this stage, methane produced by ruminal fermentation of silage can be increased.

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