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The Evaluation of γ-Zein Reduction Using Mass Spectrometry—The Influence of Proteolysis Type in Relation to Starch Degradability in Silages

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Abstract: The starch availability and nutritional value of corn (*Zea mays* L.) are affected by zein proteins. The aim of the study was to see whether the proposed reduction of γ-zeins during the fermentation of silages is a result of either the enzymatic proteolytic activity or of the acidic environment, and how this reduction affects starch availability and degradability in high-moisture corn. A mass spectrometry (MS) technique was used to quantify the 16- and 27-kDa γ-zeins. Briefly, two-dimensional gel electrophoresis (2-DE) was used for γ-zein separation, followed by densitometry for protein quantification and matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF/TOF) for protein identification. The results show that the reduction in γ-zeins induced by the ensiling led to a more pronounced starch availability and in vitro degradation, and this reduction was dependent on the type of proteolysis. More specifically, the results indicate that the reduction of γ-zeins in the ensiled corn was primarily driven by the enzymatic proteolysis. Furthermore, we demonstrated that 2-DE followed by densitometric quantification and the mass spectrometry analysis for protein identification can be used as a state-of-the-art method for γ-zein evaluation both in fresh and fermented/ensiled corn samples.

Keywords: 16–27 kDa γ-zeins; in vitro starch degradability parameters; high-moisture corn; *Lactiplantibacillus plantarum*; lactic acid; MALDI mass spectrometry

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1. Introduction

Corn (*Zea mays* L.), which is the leading cereal by world production [1], is an important source of starch. Due to the fact that starch is the primary energy source in animal feeds, corn is positioned as the most-used cereal in animal nutrition today. In corn grain, starch is located in the corn endosperm, where starch granules are surrounded with zeins, the most abundant corn proteins located in the protein bodies (PB) [2]. Zein proteins are an important feature of the corn's nutritional value and industrial potential [3–6]. They are important because they act as a physical barrier to starch digestion both by the rumen microbes [7] and in monogastric animals [8]. The more abundant zein proteins surrounding starch granules are associated with lower starch digestibility, and conversely, the sparsest zein proteins are associated with higher starch digestibility [7,8]. Furthermore, although zeins are a source of protein in human diets and animal nutrition, they have a negative N balance [5] deficient in the essential amino acids Lys and Trp [9].

For this reason, high-lysine corn mutants with a reduced zein content were developed. However, this type of corn had impaired grain characteristics with an undesirable soft and brittle endosperm, so its use was restricted due to poor agronomic performance [10].

Later on, a quality protein maize (QPM) with regained vitreous endosperm and with the agronomic performances of normal corn was developed. This new corn had a higher lysine content and a lower total zein content, but interestingly, also a higher γ-zein content [11]. This suggests that γ-zeins are essential for the PB functionality and recovered hardness in QPM [12].

Gamma zeins are the second most abundant group of zein proteins after α-zeins, accounting for 20–25% of the total zein proteins [13]. They are located in the outer regions of the PBs and are responsible for the number and characteristics of the PBs [2,14]. There are three distinctive γ-zeins that have been defined: the 16-kDa, the 27-kDa, and the 50-kDa γ-zeins, with 50-kDa γ-zeins occurring in a low abundance and have long been misidentified as a dimer of the 27-kDa γ-zein [15]. Holding and Larkins proposed that the 50-kDa and the 27-kDa γ-zeins, located at the periphery of the PBs, enable nucleation and growth of the PBs, whereas the 16-kDa enables the retention of α/δ zein proteins in the inner region of the PBs, by being physically linked to the 50-kDa and 27-kDa γ-zeins [2].

Silage production is an important feed fermentation and preservation process in which epiphytic lactic acid bacteria produce organic acids, mainly lactic acid, which reduces the pH and inhibits the activity of the spoilage microorganisms. The fermentation of green mass for silage production is called ensiling. Inoculants and chemical agents are often added as silage-making aids for fast pH decrease [16]. Lactic acid bacteria belonging to the genera of *Lactobacillus*, *Streptococcus*, and *Pediococcus* are the most common types of inoculants used for ensiling [17]. The ensiling of corn results in a higher starch degradability in the rumen, which is mainly influenced by the fate of zein proteins during the ensiling [4,18]. Corn silage production, more specifically high-moisture corn and whole-plant corn silage, results in reduced zein amounts [19,20]. In addition, the use of inoculants in the whole-plant corn silages increases zein reduction, and this reduction is primarily driven by the enzymatic proteolysis rather than by the chemical action of lactic acid in the ensiled material [20]. Because of the fundamental influence of zeins on the starch in corn grain, it is important to understand the fate of zeins during the ensiling. However, the present literature lacks evidence on the effects of ensiling on γ-zein reduction, and even more on whether the reduction is driven either by the enzymatic proteolytic activity or by the chemical action of the acidic environment found in silages [19]. As in the case of QPM, where a lower total zein content is associated with a higher γ-zein content, the fate of γ-zeins does not always correspond to the fate of the total zein amount [11].

Zein protein analysis is complex and involves the application of various techniques commonly used in protein investigation. For example, zein analysis methods include the combination of electrophoresis methods, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional (2-D) gel electrophoresis (2-DE) [12,21–23]. These methods can also be combined with the use of mass spectrometry (MS) for protein identification [23,24] or liquid chromatography-mass spectrometry (LC-MS), as applied in 2020 [25]. However, none of the above methods have been used for the study of γ-zeins so far.

The objective of this study was to evaluate the extent of an individual γ-zein reduction in the fermented/ensiled high-moisture corn using MS and densitometry. Since MS is an elementary tool for protein primary structure analysis, the standard approach of coupling MS with 2-DE was the chosen method used to reveal γ-zein changes both in the fresh and the high-moisture corn samples. To assess whether the γ-zein reduction is driven primarily either by the enzymatic proteolytic activity or by the chemical action of the acidic environment, three types of the ensiled corn were studied and they included: corn ensiled with the *Lactiplantibacillus plantarum* additive (mainly enzymatic proteolysis), corn ensiled with the lactic acid additive (mainly chemical proteolysis), and corn ensiled without any additives (standard ensiling conditions). In addition, the evaluation of the parameters of in vitro dry matter and starch degradability was carried out, since zein proteins affect starch availability.

2. Materials and Methods

2.1. Fresh and High-Moisture Corn Samples

The corn used in the study (Bc 462, FAO 420; BC Institute d.o.o., Zagreb, Croatia) was grown in five replicates (plots) under intensive conditions in the split-plot experimental fields at the University of Zagreb, Faculty of Agriculture, Zagreb, Croatia. Each plot measured 14 m^2 and was fertilized with 400 kg/ha NPK 7-20-30 (N-P2O5-K2O). The starting fertilizer was urea (100 kg/ha), and KAN N (MgO) was applied twice during vegetation (175 kg/ha). At the time of physiological maturity (70% DM), the corn was harvested and each of the replicates was ground in a hammer mill with an 8 mm sieve (Ino Brežice d.o.o., Krška vas, Slovenia). The milled material from each of the five replicates was divided into three parts for the ensiling. The first part was treated with the LAB additive Bio-Sil (Dr. Pieper Technologie und Produktentwicklung GmbH, Wuthenow, Germany), the second part with the lactic acid additive, and the third part was ensiled without any additives. Before the ensiling, 500 g of each of the five replicates was stored at −20 °C to determine the in vitro dry matter and starch degradability and the zein quantification in the fresh corn sample.

The fermentation/ensiling was performed in airtight nylon bags measuring 280 × 360 mm (Status d.o.o., Metlika, Slovenia), each containing approximately 1000 g of ground corn ensiled with or without additives. The additive Bio-Sil LAB contains two strains of *Lactiplantibacillus plantarum* and was used at a concentration of 300,000 CFU/g according to the manufacturer's instructions. The lactic acid additive was applied at a concentration of 15 g/kg DM and based on an average lactic acid content in a high-moisture corn [4]. After application, the bags were vacuum sealed using a SmartVac vacuum sealer (Status d.o.o., Metlika, Slovenia). The silages were maintained at 25 ± 2 °C and sampled after 364 days. After sampling, the high-moisture corn samples were stored at −20 °C until the zein, in vitro dry matter and starch degradability were determined.

2.2. Degradability Determination

The macro in vitro method using an Ankom DaisyII incubator (ANKOM Technology, Macedon, NY, USA) was used to determine the in vitro dry matter and starch degradability kinetics in the fresh and high-moisture corn [26]. The samples (0.7 g) were weighed in R510 bags (ANKOM Technology, Macedon, NY, USA), sealed, and placed in DaisyII incubator jars (25 bags per jar) containing 2000 mL of the incubation solution prewarmed to 39 °C. The incubation solution was prepared as described in Goering and van Soest [27] by mixing buffer solution and rumen fluid; both were prepared under CO_2 to obtain anaerobic conditions. The rumen fluid was collected from three dry cows of similar constitution and production level fed ad libitum with corn silage-based TMR. The animals were handled in accordance with the University of Zagreb Faculty of Agriculture Bioethical Committee Approval (KLASA: 114-04/22-03/06; URBROJ: 251-71-29-0111-22-4) for the protection of animals used for scientific purposes. The rumen fluid was first sieved through four layers of cheesecloth and then added (400 mL) to each jar containing 1600 mL of the buffer solution.

After 0, 7, 12, 24, 32, and 48 h of incubation, the bags were washed in ice-cold water to remove bacterial debris and dried overnight at 60 °C. All repetitions of the ensiled and fresh corn for each of the incubation periods were incubated in the same incubation jar. The dry weight of each sample before and after incubation was used to determine the in vitro dry matter degradability parameters. For the determination of the starch degradability parameters, the dried samples after the incubation in vitro were ground through a 1 mm sieve and analyzed enzymatically for starch content using the K-TSTA assay kit (Megazyme International, Bray, Ireland). The amount of starch in each sample before and after the incubation was used to determine the in vitro starch degradability parameters.

2.3. Data Analysis

The in vitro dry matter and starch degradability parameters were fitted to an experimental model according to the method described by Orskov and McDonald [28], later implementing the lag phase described in Dhanoa [29]:

$$D(t) = a + b \times (1 - e^{-c \times (t - L)})$$

In the formula, D(t) is the proportion (g/g) degraded in the time t (h), (a) is the proportion of the rapidly degraded fraction (g/g), (b) is the proportion of the potentially degraded fraction (g/g), (c) is the degradation rate constant (h^{-1}), and L is the duration of the lag phase (h).

The degradation parameters were provided using the non-linear least squares regression procedure NLIN in SAS 9.3 (SAS Institute, Cary, NC, USA) with the Marquardt method to obtain the smallest residual sum of squared deviations from the model.

The effective degradability (ED) was determined according to Peyrat et al. [30], where ruminal outflow (kp, h^{-1}) is defined as 0.04 for dry matter degradation and 0.06 for starch:

$$ED = a + (b \times ce^{(-kp \times L)})/(c + kp)$$

The effects of ensiling, the ensiling additive, and their interactions on the in vitro degradability parameters were analyzed using the PROC MIXED procedure of SAS 9.3. The degradability parameters were reported as a mean ± standard deviation of five repetitions. The ensiling was treated as a repeated statement and the ensiling additive as a random effect. The means were defined using least squares means statement and compared with the PDIFF option. Letter groups were defined using the PDMIX macro in SAS [31]. The differences and interactions were considered significant when $p < 0.05$.

2.4. Gamma Zein Evaluation

2.4.1. Zein Extract Preparation

Zein proteins from the fresh and high-moisture corn samples were extracted as described in Wu et al. [12], with some modifications. Corn samples of 150 mg in 2 mL Eppendorf Safe-Lock tubes (Eppendorf, Hamburg, Germany) were thoroughly mixed at 25 °C for 2 h with 1.2 mL of the extraction buffer (70% ethanol/2% 2-mercaptoethanol, vol/vol) on a thermomixer (Thermomixer R, Eppendorf, Hamburg, Germany). After mixing, the samples were centrifuged at 4 °C for 10 min at 12,000 rpm (Eppendorf 5415R refrigerated centrifuge, Eppendorf, Hamburg, Germany) and 100 µL of the supernatant was used for zein precipitation with acetone overnight at −20 °C. The precipitated zein protein samples in acetone were centrifuged at 4 °C for 20 min at 12,000 rpm, and the zein residues were resuspended in 100 µL of the rehydration solution for isoelectric focusing (IEF). The protein concentration in the resuspended samples was determined by Bradford protein assay [32] and the same amount of total proteins per sample was separated by 2-D gel electrophoresis. Next, the separated proteins were determined by the negative chemically activated fragmentation/positive chemically activated fragmentation (CAF−/CAF+) technique for protein identification as described in Butorac et al. [33], with some modifications. The procedure is illustrated in Figure 1 and described below.

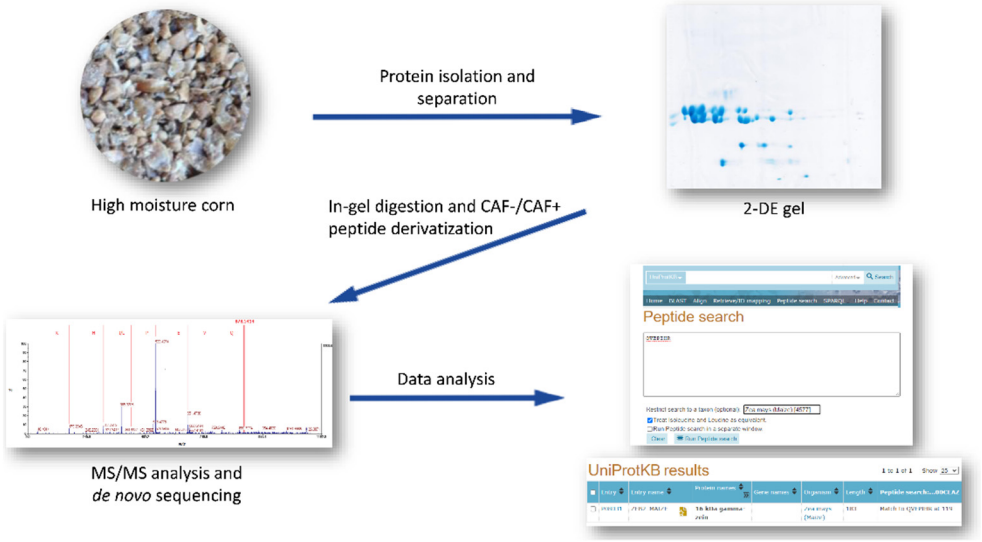


Figure 1. Schematic representation of chemically activated fragmentation (CAF−/CAF+) mass spectrometry (MS) technique for protein identification. From top left to bottom right: zein proteins isolated from the fresh and the high-moisture corn samples were separated on 2-DE gels, visualized with Coomassie brilliant blue, and analyzed with PDQuest 2-D image analysis software. All visible spots were excised from the gels, processed by in-gel digestion and CAF−/CAF+ peptide derivatization and analyzed using the MS technique for protein identification.

2.4.2. Two-Dimensional (2-D) Gel Electrophoresis with Zein Quantification

Linear immobilized pH gradient strips (17 cm, pH 3–10) on a Protean IEF cell (Bio-Rad, Hertfordshire, UK) were used for protein separation by IEF in the first dimension. The second dimension, SDS-PAGE electrophoresis, was performed on 1 mm thick 12% polyacrylamide gels with protein visualization using Coomassie brilliant blue.

Protein quantification based on the intensity of visible protein spots stained with Coomassie brilliant blue was performed using image analysis software (Discovery Series PDQuest 2-DE analysis software package, version 7.4.0) integrated with a VersaDoc 4000 Imaging System (Bio-Rad, Hertfordshire, UK). The expression difference evaluated with the image analysis software was statistically significant at $p < 0.05$ [34].

2.4.3. Sample Preparation for Mass Spectrometry

The labeled and quantified spots obtained after Coomassie brilliant blue staining were excised and subjected to tryptic in-gel digestion. After protein digestion, the peptides were resuspended in 10 µL of 0.1% TFA solution, purified on C4 ZipTip columns, and evaporated to dryness in a vacuum centrifuge at 30 °C (Concentrator Plus complete system, Eppendorf, Hamburg, Germany). The dried samples were dissolved in the derivatization solution CAF−/CAF+ and the derivatization was performed in three cycles for 4 min at 90 W in a microwave oven. Thereafter, C4 ZipTip purification and vacuum centrifuge drying were performed again. The dried peptide samples were dissolved in 5 µL of a 5 mg/mL CHCA matrix prepared in a water/acetonitrile mixture (1:1, *v*/*v*) and spotted onto a metal matrix-assisted laser desorption/ionization (MALDI) plate.

2.4.4. Mass Spectrometry and Protein Identification

Mass spectrometric analysis was performed using a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems Inc., Foster City, CA, USA) equipped with a 200 Hz, 355 nm Nd/YAG laser. The mass spectra were obtained by averaging 1800 laser shots covering a mass range from m/z 800 to 5000. Acquisitions of derivatized peptides were performed in the negative ion mode; the internal calibration of the mass range was performed using derivatized tryptic autolysis fragments. Gamma zein proteins were digested in silico to

generate a list of potential peptide ions derived from γ-zein proteins. The generated list was used for γ-zein detection by the MS/MS analysis. In parallel, the 10 most intense precursor signals from the MS negative spectra were selected for further MS/MS analysis to detect other proteins present in the samples. The MS/MS analysis was performed in negative and positive ion modes at 1 kV collision energy with air as a collision gas. Protein identification and database searches of derivatized peptides were performed using Protein Reader (SemGen Ltd., Zagreb, Croatia) against the NCBInr database.

2.5. Scanning Electron Microscopy Imaging of Ground Corn Samples

The ensiling and ensiling additive application impact on starch and protein interactions (starch–protein matrix) in the high-moisture corn grains were evaluated visually on 1 mm ground samples using a scanning electron microscope (SEM) (FE-SEM, Mira II LMU, Tescan, Brno, Czech Republic). For this purpose, all repetitions from each treatment were pooled. The SEM operated at 5 kV under magnifications of 2000×, 5000×, or 10,000×. Prior to the SEM analysis, the samples were sputter coated with Cr alloy (Q150T ES Plus, Quorum Technologies, Lewes, UK).

3. Results

3.1. In Vitro Degradability Results

The ensiling increased the dry matter and starch degradation rates compared to the fresh corn sample (for DM $p = 0.002$, for starch $p < 0.001$; Table 1). However, the results varied depending on the ensiling additive. A significant increase in the dry matter and starch degradation rate of the high-moisture corn ensiled with the lactic acid additive was not observed compared to the fresh corn sample ($p > 0.05$), although the starch degradation rate value in the ensiled corn was 21% higher than that of the fresh corn sample. Silages ensiled with *Lactiplantibacillus plantarum* inoculant had a 59% higher dry matter degradation rate and a 160% higher starch degradation rate compared to the fresh corn sample ($p < 0.05$).

Table 1. Effects of ensiling and ensiling additives on in vitro dry matter (DM) and starch degradability parameters.

		High-Moisture Corn			*p*		
Kinetics Parameters	**Fresh Corn**	**Inoculant *L. plantarum***	**Lactic Acid**	**Control**	**Ensiling (E)**	**Additive (A)**	**E×A**
			DM [6] degradability parameters				
ED [1]	0.59 [b]	0.83 [a]	0.76 [a]	0.80 [a]	<0.001	0.302	0.302
a [2]	0.28 [b]	0.58 [a]	0.52 [a]	0.58 [a]	<0.001	0.579	0.579
b [3]	0.69 [a]	0.47 [b]	0.47 [b]	0.46 [b]	<0.001	0.995	0.995
c (h^{-1}) [4]	0.0561 [b]	0.0892 [a]	0.0453 [b]	0.0782 [a]	0.002	0.003	0.001
lag (h) [5]	7.28 [a]	4.01 [b]	3.33 [b]	6.02 [a]	<0.001	0.142	0.142
			Starch degradability parameters				
ED [1]	0.56 [b]	0.83 [a]	0.78 [a]	0.79 [a]	<0.001	0.529	0.530
a [2]	0.36 [c]	0.64 [ab]	0.60 [b]	0.68 [a]	<0.001	0.214	0.214
b [3]	0.63 [a]	0.37 [b]	0.41 [b]	0.33 [b]	<0.001	0.693	0.693
c (h^{-1}) [4]	0.0527 [b]	0.1368 [a]	0.0638 [b]	0.0835 [b]	<0.001	0.035	0.024
lag (h) [5]	6.54 [a]	3.03 [b]	2.34 [b]	5.44 [a]	0.003	0.002	0.002

[1] ED: effective degradability. [2] a: rapidly degradable fraction. [3] b: potentially degradable fraction. [4] c: rate of degradation (h^{-1}). [5] lag: lag phase (h). [6] DM: dry matter. alues with different superscript letters in the same row represent differences ($p < 0.05$) between samples [31].

The dry matter and starch effective degradability were higher (both $p < 0.001$) in the high-moisture corn than in the fresh corn (Table 1). This was mainly related to the DM and starch degradation rate as well as to the DM and starch rapidly degradable fraction, which were higher in the silages than in the fresh corn ($p < 0.001$). In contrast to the DM and starch degradation rate, the ensiling additive showed no significant effect on the DM and starch rapidly degradable fraction (for DM $p = 0.579$, for starch $p = 0.214$; Table 1). Consistent

with the increase in the DM and starch rapidly degradable fractions, the DM and starch potentially degradable fractions decreased with the ensiling ($p < 0.001$, Table 1).

Similar to all of the degradability parameters evaluated, the lag phase showed an improvement with the ensiling ($p < 0.001$ for DM, $p = 0.003$ for starch); both the DM and starch lag phases were shorter in the high-moisture corn, with the lag phase being the shortest in the high-moisture corn ensiled with the additives ($p < 0.05$; Table 1). The high-moisture corn ensiled without the additives also showed a shorter lag phase, but with no significant improvement compared to the fresh corn ($p > 0.05$).

At all of the time points examined, the disappearance of DM and starch were more intense in the high-moisture corn than in the fresh corn ($p < 0.05$), with the highest values observed in the high-moisture corn ensiled with *Lactiplantibacillus plantarum* (Figure 2). The highest peak values for both the DM and starch disappearances in the high-moisture corn samples were at 7 h of incubation, whereas fresh corn had the highest DM and starch disappearance peak values at 12 h of incubation. As shown in Figure 2, the high-moisture corn silages had values twice as high for the disappearance of DM (average from 33.40% to 67.99%) and 66% higher values for the disappearance of starch (average from 46.15% to 76.57%) from the beginning to 7 h of incubation, while they had a similar disappearance of DM and starch at 12 h compared to 7 h of incubation (Figure 2). However, in the fresh corn samples, the disappearance of DM was 48% and the disappearance of starch was 40% higher from 7 h to 12 h compared to the disappearance from the beginning of incubation to the 7 h time point (Figure 2).

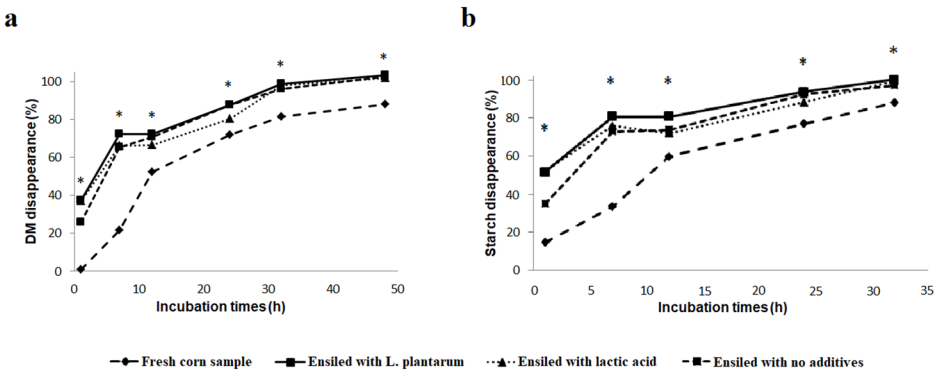
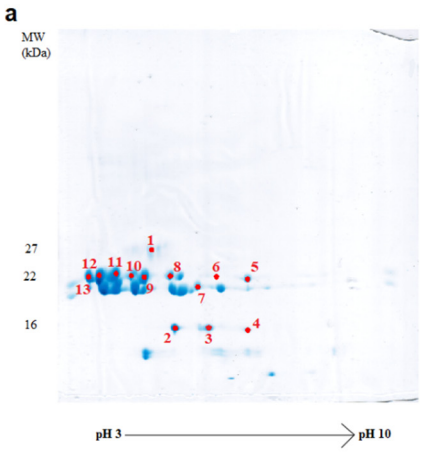


Figure 2. In vitro disappearances of DM (**a**) and starch (**b**) in the fresh corn and high-moisture corn samples. Incubation time points marked with an asterisk show significant differences between treatments ($p < 0.05$).

3.2. Zein Protein Pattern

On each of the 2-DE gels of zein extracts from the fresh and high-moisture corn, more than 50 protein spots were visualized with Coomassie brilliant blue (Figures 3 and 4). All detected protein spots were excised from the gels, analyzed by mass spectrometry, and later identified using the bioinformatics software Protein Reader. A total of six zein proteins were identified in 13 spots (Figure 3) and their positions on each of the 2-DE gels were indicated (Figure 4). Of the 13 identified zein spots, four were identified as γ- zeins. Three of them contained the 16-kDa γ-zeins, while the remaining one contained the 27-kDa γ-zein. (Figures 3 and 4).

When comparing the 2-DE gels of fresh corn with the high-moisture corn samples, protein degradation in the ensiled corn samples was visible as protein smears on the 2-DE gels (Figure 4b–d gels). The exact degree of γ-zein reduction (degradation) in the high-moisture corn samples was determined using the image analysis software. The difference in the expression evaluated with the image analysis software was statistically significant ($p < 0.05$, Figure 5).



Spot location	Protein	NCBI Accession No.
1	27 kDa γ-zein	CAA41175
2	16 kDa γ-zein	ABD63256
3	16 kDa γ-zein	ABD63256
4	16 kDa γ-zein	ABD63256
5	22 kDa zein	AAA33542
6	22 kDa zein and M36M361 zein	AAA33542 and 1107201E
7	22 kDa zein	AAA33542
8	M36M361 zein	1107201E
9	M36M361 zein	1107201E
10	M36M361 zein	1107201E
11	M36M361 zein	1107201E
12	z1A α-zein	ABV71937
13	M36M361 zein	1107201E

Figure 3. Zein protein separation on 2-DE gel. Detected zeins were visualized using Coomassie brilliant blue (**a**) and labelled by numbers (red), with indicated corresponding NCBI Accession numbers (**b**).

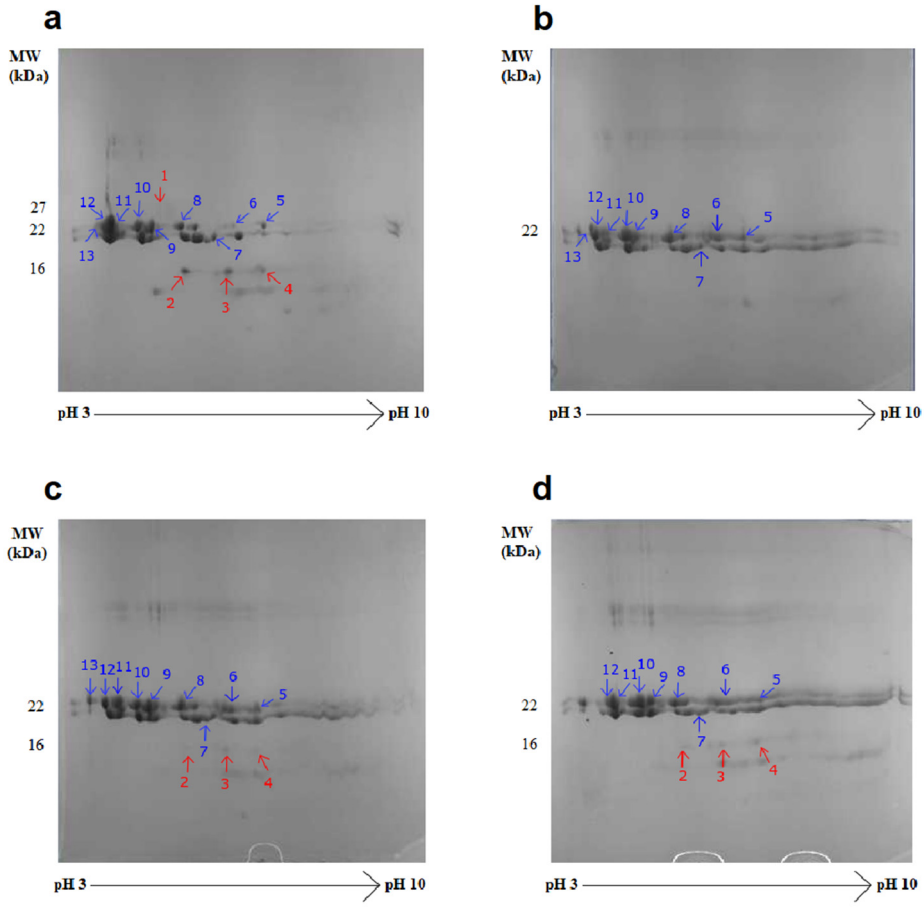


Figure 4. Detection of zein proteins. The proteins from fresh corn (**a**), high-moisture corn ensiled with inoculant *Lactiplantibacillus plantarum* (**b**), lactic acid (**c**), and without additives (**d**) were separated on 2-DE using linear immobilized pH gradient strips (17 cm, pH 3–10) in the first and 12% polyacrylamide gels in the second dimension. All visible spots were excised from the gels and analyzed by mass spectrometry. Identified γ-zein protein spots are indicated by red numbers and other zein protein spots by blue numbers. The numbers correspond to zein proteins defined as in Figure 3b.

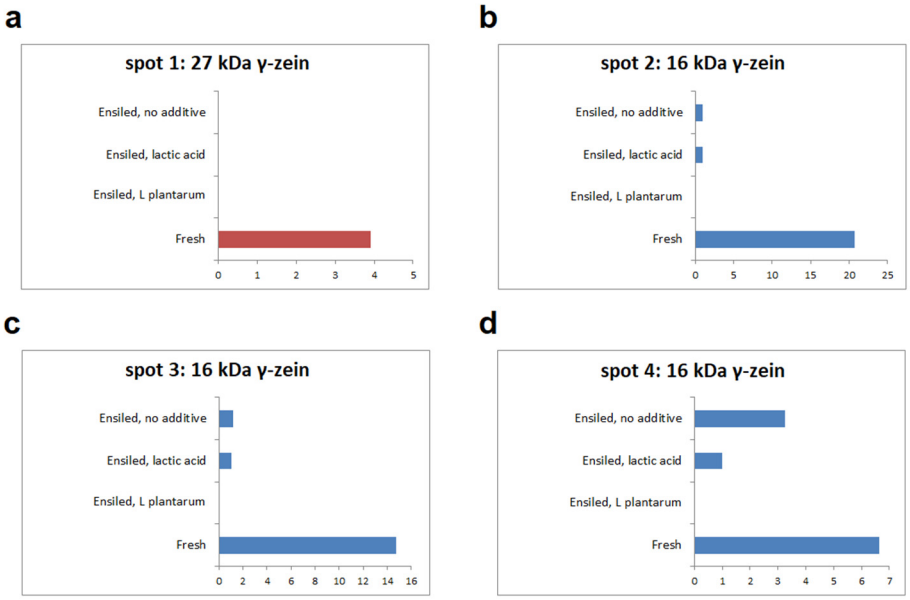


Figure 5. Evaluation of differences in detected γ-zein proteins. The proteins were analyzed with image analysis PDQuest 2-D software depending on the ensiling treatment: 27 kDa γ-zein spot 1 (**a**), 16 kDa γ-zein spot 2 (**b**), 16 kDa γ-zein spot 3 (**c**), and 16 kDa γ-zein spot 4 (**d**). The expression difference is statistically significant at $p < 0.05$ [34].

3.3. γ-Zein Reduction

The results of the γ-zein changes in the fresh and high-moisture corn samples (Figure 5) showed a reduction in γ-zein proteins in the high-moisture corn. The ensiling of high-moisture corn with *Lactiplantibacillus plantarum* inoculant did not result in detectable γ-zein proteins (Figures 4 and 5). In contrast, the ensiling of high-moisture corn with the lactic acid and without the addition of the additives resulted in no detectable 27-kDa γ-zein and detectable levels of the 16-kDa γ-zein. The reduction in the 16-kDa γ-zein was, on average, 45% greater in the high-moisture corn ensiled with lactic acid than in the high-moisture corn ensiled without the additives (Figure 4; $p < 0.05$, Figure 5).

4. Discussion

The main objective of the study was to evaluate γ-zein proteins in fresh and high-moisture corn samples by adapting the 2-DE separation method coupled with MALDI peptide mass fingerprinting, which Postu et al. [23] used to evaluate α-zeins extracted from fresh corn. In the present study, for the first time, γ-zein proteins in fresh and ensiled corn were evaluated using 2-DE followed by densitometry quantification and MALDI-TOF/TOF for protein identification. Gamma zeins, the second most abundant group of zein proteins, are essential for the PB functionality and abundance [14]. Since starch granules are surrounded by the PBs in the starch–protein matrix [2], the starch availability and nutritional value of corn are strongly associated with their number and properties [4,35,36]. Therefore, in addition to γ-zein evaluation, in vitro starch degradability was another important subject of this study.

In the present study, extraction with a reducing agent in aqueous alcohol [12] was used, differing from the extraction method of Postu et al., who extracted zeins in 65% and 95% ethanol [23]. Gamma zeins are Cys-rich proteins that exhibit strong disulfide bonds, which affect the stability and extractability of γ-zeins [13]. Therefore, the extractability of γ-zeins is low without the use of a strong reducing agent [37], whereas aqueous alcohol is sufficient for the extraction of α-zeins [3,23]. In addition, all four types of zeins are extractable in aqueous alcohol solution with a reducing agent [3]. Here, the observed simultaneous extraction of γ-zeins and α-zeins was not a surprise due to the strong protein–protein

interactions between the γ- and α-zeins, with the former located at the periphery of the PB and the latter in the center of the PB under the γ-zein layer [2,38].

Zein extracts from the fresh and high-moisture corn samples separated on 2-DE had four γ-zein spots and nine α-zein spots, whilst the other detected proteins were of non-zein origin. Some of the non-zein protein spots were detected at the same molecular weight as the zeins, which implies that some zein proteins might actually not be zein proteins when zein detection is based merely on the molecular weight markers usually implemented with SDS-PAGE zein detection [12,21–23].

Proteolytic enzymes and an acidic environment are responsible for protein degradation in silages [16], which is also clearly seen here. The results show a reduced protein layer/coating around the starch granules in the fermented/ensiled high-moisture corn compared to the fresh corn samples when the samples were visualized with SEM (Figure 6). The fermentation end-products during ensiling, namely, lactic and acetic acids, are good zein solubilizers [3], but in the ensiled whole-plant corn, zein degradation is mainly driven by the enzymatic proteolysis rather than by the chemical action of lactic acid [20]. Junges et al. further confirmed the enzymatic nature of protein breakdown in reconstituted corn grain silages [39]. The authors compared the contribution of the corn kernel, bacteria, fungi, and fermentation end-products to protein breakdown and concluded that fermentation end-products only made a minor contribution, while bacterial enzymes (60%) and corn kernel enzymes (30%) had the greatest impact on protein breakdown [39]. Similarly, Hoffman et al. showed that ensiling leads to the degradation of γ-zein in high-moisture corn, but, in contrast to the results presented here, with no difference in the extent of reduction when the inoculant was used [19]. The results of this study supported the enzymatic proteolysis reported by Duvnjak et al. [20] and Junges et al. [39] and showed that zein reduction, more specifically the 16-kDa γ-zein reduction, was strong in the bacteria-inoculated silages, moderate in the silages with the application of the lactic acid additive, and the lowest when there were no additives applied. The changes in the 27-kDa γ-zein during the ensiling showed a complete reduction of this γ-zein protein type. The observed differential intensity of the reduction of the 27-kDa γ-zein and the 16-kDa γ-zein was consistent with the proposed locations of both types of γ-zeins; the 27-kDa are localized at the periphery of the PB and the 16-kDa are directed towards the inner regions of the PB, and are responsible for the retention of α/δ zein proteins in the inner region of the PB [2].

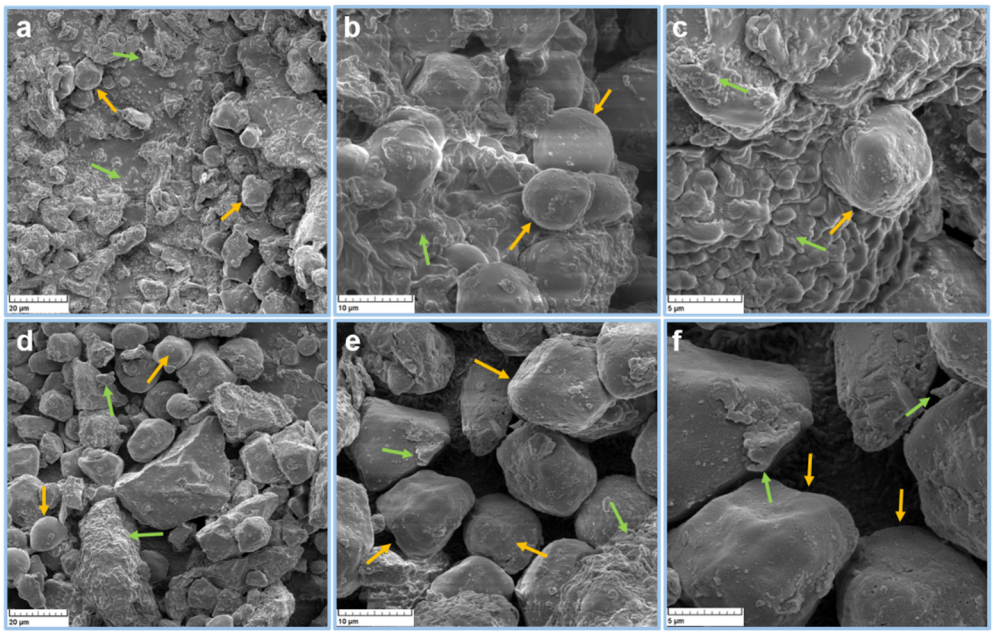


Figure 6. Scanning electron micrographs representing differences in the starch–protein matrix of fresh and ensiled high-moisture corn. The differences were examined on 1 mm ground corn samples

using a scanning electron microscope operating at 5 kV under 2000×, 5000×, or 10,000× magnifications. Scanning electron micrographs are as follows: fresh corn 2000× (**a**), fresh corn 5000× (**b**), fresh corn 10,000× (**c**), ensiled high-moisture corn 2000× (**d**), ensiled high-moisture corn 5000× (**e**) and ensiled high-moisture corn 10,000× (**f**). Starch granules in the ensiled samples (**d**–**f**) were more accessible due to the reduction of the zein protein layer surrounding the starch granules compared to the fresh corn samples (**a**–**c**). On scanning electron micrographs, examples of starch granules are marked with orange arrows and examples of zein protein coating/layer are marked with green arrows.

The PBs are not randomly, but rather uniformly distributed around the starch granules embodied in the protein matrix. In general, their interaction with the surrounding cytoskeletal network is influenced by the appropriate location and proportions of the zeins, especially the γ-zein proportions, as they are located at the periphery of the PBs [12]. The in vitro degradability data from this study support Hoffman et al. [19], who showed that the reduction of γ-zeins during the ensiling leads to a disassociation of starch granules and thus allows greater access to starch granules by the rumen microorganisms. In the present study, the intensity of γ-zein reduction (Figures 4 and 5) and the in vitro degradability results (Table 1), as well as the visual representation of the morphological changes of the starch–protein matrix (Figure 7), were in agreement with each other. The in vitro starch and dry matter degradability were higher in the high-moisture corn than in the fresh corn. These results are in agreement with the in situ ruminal degradability parameters in ensiled corn grain [18], ensiled whole-plant corn [30], ensiled steam-flanked corn TMR [40], and the in vitro ruminal degradability in ensiled whole-plant corn [41]. For the ensiled whole-plant corn, the authors reported a higher degradation rate and effective degradability, and a higher content of rapidly degradable fraction in the ensiled compared to the fresh material. The increase in the in vitro degradability of the DM and starch in the high-moisture corn samples was mainly due to the increase in the rapidly degradable fraction [18]. The data indicate that the high-moisture corn ensiled with *Lactiplantibacillus plantarum* showed the highest degradability rate of both DM and starch, which corresponds to γ-zein reduction and thereby with the microenvironment that allows the more intense activity of amylolytic microorganisms [42,43]. The visual representation of the changes in the starch–protein matrix using SEM is consistent with the above. As seen from the scanning electron micrographs, the protein layer was reduced and the starch granules were dissociated in the ensiled high-moisture corn (Figure 6), with the greatest reduction observed in the high-moisture corn ensiled with *Lactiplantibacillus plantarum* (Figure 7).

Ruminal degradability is defined by the first-order kinetics equation, the so-called McDonald model [28], which usually neglects the influence of the lag phase by assuming immediate substrate hydration and colonization by microorganisms [44]. However, Dhanoa [29] showed that the simultaneous estimation of the lag phase parameter within the McDonald model is beneficial for the adequate evaluation of the kinetic parameters. In this study, the ensiling affected the lag phase of both starch and DM degradability; the reduction in their duration was consistent with the proposed influence of zein on starch availability. The high-moisture corn silages ensiled with *Lactiplantibacillus plantarum* and lactic acid that exhibited a more intense γ-zein reduction also had shorter lag phases. The control silages with less intensive γ-zein reduction had longer lag phases with no significant differences compared to the fresh corn.

When comparing the disappearance of starch and DM at different incubation times, the high-moisture corn silages showed different disappearance patterns than the fresh corn. The silages had a significantly higher and earlier disappearance of both DM and starch compared to the fresh corn, which supports the proposed greater access to starch granules by the rumen microorganisms in ensiled corn [19]. As the zein proteins are degraded, the starch granules become more accessible, consequently leading to an earlier disappearance of starch, an effect seen in the SEM analysis performed here (Figures 6 and 7). The scanning

electron micrographs showed that the starch granules in the ensiled samples were more accessible due to the reduction of the zein protein layer surrounding the starch granules.

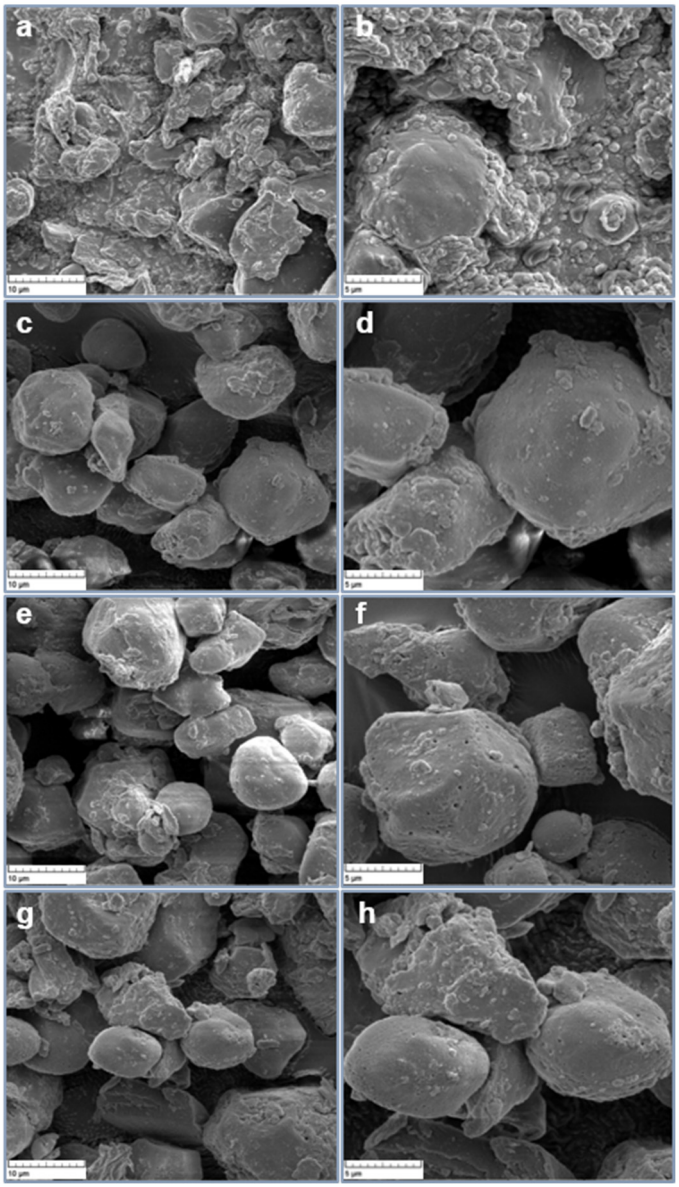


Figure 7. Scanning electron micrographs of zein protein degradation in the three types of ensiled high-moisture corn. Degradation of zein proteins in the ensiled high-moisture corn samples compared to the fresh corn samples was examined on 1 mm ground samples using a scanning electron microscope operating at 5 kV under 5000×, or 10,000× magnifications. Scanning electron micrographs of ground corn samples are as follows: fresh corn 5000× (**a**), fresh corn 10,000× (**b**), high-moisture corn ensiled with inoculant *Lactiplantibacillus plantarum* 5000× (**c**), high-moisture corn ensiled with inoculant *Lactiplantibacillus plantarum* 10,000× (**d**), high-moisture corn ensiled with lactic acid 5000× (**e**), high-moisture corn ensiled with lactic acid 10,000× (**f**), high-moisture corn ensiled without additives 5000× (**g**), and high-moisture corn ensiled without additives 10,000× (**h**). The zein protein layer in the ensiled high-moisture corn was reduced compared to the fresh corn; the highest reduction was in the *Lactiplantibacillus plantarum*-ensiled high-moisture corn.

5. Conclusions

This study clearly shows that the extent of γ-zein reduction in high-moisture corn depends on the type of additive, and that the reduction was primarily caused by the enzy-

matic proteolysis. The more intense γ-zein reduction resulted in higher starch availability and degradability, demonstrating the importance of γ-zein to the nutritional value of corn.

Furthermore, the results of this study demonstrated that the combination of 2-D gel electrophoresis followed by the densitometry quantification and MALDI-TOF/TOF for protein identification, which was applied here, can be used as a state-of-the-art method for γ-zein evaluation in fresh and ensiled corn samples.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the use of a commercial maize hybrid in the study and the intellectual property rights of the hybrid producer.

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