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Short communication: Influence of various proteolytic sources during fermentation of reconstituted corn grain silages

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ABSTRACT

The objective of this study was to determine the contribution of corn kernel enzymes, bacteria, fungi, and fermentation end-products (main acids and ethanol) to protein solubilization during fermentation of reconstituted corn grain silage. Flint corn kernels were ground (5-mm sieve), rehydrated to 32% of moisture, and treated with no additives (control), gamma irradiation (32 kGy), gamma irradiation + fermentation end-products (1% of lactic acid, 0.3% of acetic acid, and 0.7% of ethanol, as fed), and natamycin (1% as fed). Treated grains were ensiled in nylon-polyethylene bags and stored for 90 d. Protein solubilization was calculated for each treatment and the contributions of proteolytic sources were determined. Bacterial activity was the main contributor to proteolysis (60%) followed by corn kernel enzymes (30%), whereas fungi and fermentation end-products had only minor contributions (∼5% each).
Key words: fermentation, prolamin, protein matrix, starch digestibility

Short Communication

The starch-protein matrix in corn kernels is a physicochemical impairment to starch digestion in ruminants (Owens et al., 1986). Ensiling corn grain with high moisture content causes degradation of endosperm hydrophobic proteins during the fermentation process (Thornton, 1976; Baron et al., 1986). Several mechanisms are involved in the breakdown of proteins in whole-crop silages, such as plant enzyme-mediated as well as microbial and acid-induced proteolysis (Ohshima and McDonald, 1978; Heron et al., 1986). In corn grain silages, protein degradation can be due to kernel proteases (Simpson, 2001), microorganisms (Baron et al., 1986), and fermentation acid solubilization (Lawton, 2002), but the relative contribution of these agents to total proteolysis is unknown.

It has been claimed that fermentation acids play a major role in solubilizing the proteinaceous matrix surrounding corn starch granules and increasing starch digestibility (Prigge et al., 1976; Philippeau and Michalet-Doreau, 1998). However, substantial changes in acid load have not been associated with breakdown of zein proteins during fermentation of corn grain silage (Hoffman et al., 2011; Ferraretto et al., 2015). This suggests that the starch-protein matrix in corn kernels is more likely degraded by microbial activity rather than by simple solubilization by fermentation end-products, particularly acids. Research is needed to quantify the relative roles of these different agents of proteolysis in corn kernels to develop effective strategies for increasing protein degradation and optimizing starch digestibility.

The objective of our study was to estimate the relative contribution of corn kernel enzymes, bacteria, fungi, and fermentation end-products to protein solubilization during fermentation of reconstituted corn grain silages. As bacteria are the main microbial population in silages (Pahlow et al., 2003), we hypothesized that bacterial proteolytic activity would be responsible for most of the protein solubilization in corn grain silage, whereas fungi, fermentation end-products, and kernel enzymes could contribute to a lesser extent.

Corn kernels (∼15 kg) with flint endosperm (79.2% of vitreousness determined by manual dissection; Dombrink-Kurtzman and Bietz, 1993) were ground using a hammer mill with a 5-mm screen (ML 75-B, Lucato, Limeira, Brazil), rehydrated with distilled water to achieve a moisture content of 32%, and manually split into 4 equal piles. Pile 1 was not treated (control; **CON**); pile 2 was treated with natamycin (1% as fed; Natamax, Danisco, Copenhagen, Denmark; **NAT**) to inhibit fungal metabolism (te Welscher et al., 2008); pile 3 was treated with a solution containing a mix-

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ture of fermentation end-products to supply the main compounds formed during fermentation in corn grain silages, based on available literature (Morais et al., 2017) and previous experiments in our laboratory (1% of lactic acid, 0.3% of acetic acid and 0.7% of ethanol, as fed basis; 99% purity, Merck, Darmstadt, Germany); and pile 4 was left untreated. The natamycin and fermentation end-products were diluted in the distilled water used to rehydrate the ground kernels right before the reconstitution. Subsequently, grains from each pile were placed into each of 6 nylon-polyethylene bags (22 × 35 cm, 0.18 mm thick; Intervac, São Paulo, Brazil) and filled bags (500 g, as fed) were heat-sealed using a vacuum sealer (Jet25; Jetvac, São Caetano do Sul, Brazil). All 6 of the untreated bags from pile 4 were irradiated with 32 kGy of ^{60}Co gamma radiation for 4 h (**IRR**) at the Nuclear and Energetic Research Institute (São Paulo, Brazil). All 6 of the bags treated with the fermentation end-products from pile 3 were similarly irradiated (**IRR+FEP**). The irradiation dose was chosen because in previous reports it curtailed fermentation without adversely affecting plant enzyme activities and chemical composition (Woolford, 1983; Heron et al., 1986). All bags from the 4 treatments (CON, NAT, IRR, and IRR+FEP) were stored at room temperature (24 ± 3°C) for 90 d. Three samples of unfermented grains were frozen at −20°C for subsequent analysis.

Subsamples of unfermented grains and grain silages were dried at 60°C for 48 h, ground through a 1-mm screen using a Wiley-type mill (MA-680, Marconi, Piracicaba, Brazil) and analyzed for DM (AOAC, 1990), CP (AOAC, 1990), and soluble protein (Krishnamoorthy et al., 1982). Silage extracts were prepared by mixing 25 g of silage with 225 mL of distilled water in a stomacher for 4 min (#130/1, Nova Ética, Vargem Grande Paulista, Brazil). The solution was filtered through 2 layers of sterile cheesecloth, serially diluted in peptone water (10^{-2} to 10^{-6}), and pour-plated in agar plates for microbial counts. De Man, Rogosa, and Sharpe agar (MRS 7543A, Acumedia, Lansing, MI) supplemented with natamycin (0.25 g/L) was used for enumeration of lactic acid bacteria (incubation at 30°C for 2 d). Malt extract agar (MEA M137, Himedia, Mumbai, India) acidified with lactic acid 85% (3 mL/L) was used for enumeration of yeasts and molds (incubation at 30°C for 2 and 4 d, respectively). Afterward, tubes with serial dilutions were pasteurized (80°C for 13 min) and pour-plated in reinforced clostridial agar (RCA M154, Himedia) supplemented with neutral red (0.05 g/L) and cycloserine (0.2 g/L) for enumeration of clostridia (anaerobic incubation at 37°C for 5 d).

The pH of filtered silage extracts was measured using an electrode (DME-CV1, Digimed, São Paulo, Brazil) and an aliquot was centrifuged at 10,000 × *g* for 30 min at 5°C for analysis of fermentation end-products. Lactic acid (Pryce, 1969) and NH_3-N (Chaney and Marback, 1962) were determined by colorimetry. Ethanol and acetic and butyric acids were measured by GC-MS (GCMS QP 2010 plus, Shimadzu, Kyoto, Japan) using a capillary column (Stabilwax, Restek, Bellefonte, PA; 60 m, 0.25 mm i.d., 0.25 m).

Soluble protein was as an index of proteolysis rather than NH_3-N because the latter is an indicator of deamination, rather than proteolysis, and not all the soluble protein is deaminated during fermentation. Therefore, protein solubilization was calculated for each treatment as

$$[\text{soluble protein after fermentation (\% CP)}] - [\text{soluble protein before fermentation (\% CP)}].$$

Contributions of proteolytic sources (kernel enzymes, fermentation end-products, fungi, and bacteria) to total proteolysis were calculated as

$$\text{Kernel enzymes (\%)} = 100 \times [(\text{protein solubilization in IRR silage})/(\text{protein solubilization in CON silage})],$$

$$\text{Fermentation end-products (\%)} = 100 \times [(\text{protein solubilization in IRR+FEP silage} - \text{protein solubilization in IRR silage})/(\text{protein solubilization in CON silage})],$$

$$\text{Fungi (\%)} = 100 \times [(\text{protein solubilization in CON silage} - \text{protein solubilization in NAT silage})/(\text{protein solubilization in CON silage})], \text{ and}$$

$$\text{Bacteria (\%)} = 100 - (\text{contributions to total proteolysis from kernel enzymes + fermentation end-products + fungi}).$$

Silage composition data were analyzed for a completely randomized design using the GLM procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC). Means were compared using the Tukey test ($\alpha = 0.05$).

The unfermented grain contained 8.19 ± 0.12% of CP (% of DM), 11.6 ± 0.55% of soluble protein (% of CP), and 0.27 ± % of NH_3-N (% of N) and had a pH = 6.03 ± 0.06. The composition of the corn grain silages is shown in Table 1. The CON and NAT silages had the highest concentrations of soluble protein and NH_3-N, indicating the greatest proteolysis. This was mainly due to microbial activity (Baron et al., 1986; Hoffman et al., 2011), as indicated by the much lower